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PROTAMINE BINDING LOCATION ON DNA

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Dafna Manasseh

August 1995

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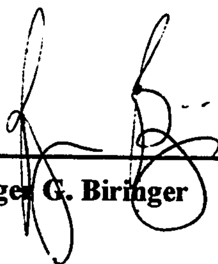

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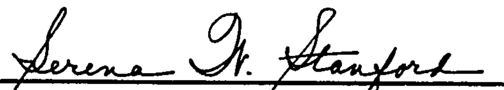

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// ABSTRACT

PROTAMINE BINDING LOCATION ON DNA

by Dafna Manasseh

Protamines form a very condensed structure with DNA, and an incredible decrease in volume occurs when a sperm cell is produced. Many methods have been used to investigate the structure of the DNA-protamine complexes. A methylation protection experiment was used in this research to determine the actual binding site of protamine P1 on B-form DNA. A complete methylation and cleavage protocol for isolated DNA-protamine complexes was developed based on the protocol by Maxam and Gilbert (1980).

Protamine P1 was complexed with [^{33}P]-labeled 12 base pair DNA and dissociation experiments with various solutions were conducted to determine the most appropriate dissociation solution. Methylation was conducted with dimethyl sulfate (DMS) which methylates the N-7 of guanine in the major groove and the N-3 of adenine in the minor groove. The cleavage of DNA at the methylated sites produced DNA fragments which were electrophoretically separated. A comparison between the cleavage products of bound DNA and free DNA revealed no differences.

It appears that the stop solution may not have effectively prevented further methylation of the DNA, thus giving rise to identical cleavage products from the free and the bound DNA. Other alternatives are also discussed, which would result in identical cleavage products.

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Introduction

The formation of DNA-protamine complexes is crucial for the production of functional vertebrate sperm cells and the dissociation of these complexes in the fertilized egg is essential for the creation of an embryo.

Protamines, which are found only in sperm cells, form a very condensed structure with the DNA (1). It is a well recognized phenomenon that the DNA's negative charge is neutralized by the protamines (2). However, the actual mechanism of this process is still under investigation. The exact binding location of the protamines on the DNA or the arrangement of the complexes have remained undiscovered and various options and models have been proposed to explain it.

The DNA-protamine complexes are very tightly bound, probably causing all or most of the water molecules present with the DNA to detach from it (3, 4). Moreover, since the sperm cell head is much smaller than a stem cell nucleus, it has been assumed by Pogany et al. (2) that the condensation is achieved by the binding of the protamines to the DNA grooves. The compact and hydrophobic structure of the complexes results in their precipitation in solutions even in very low concentrations, making it difficult to perform any work with DNA-protamine complexes in free solutions or in any other solvent system.

A methylation protection experiment (5, 6) was chosen as the subject of this thesis to investigate the binding site of the protamine on the DNA double helix, and to determine whether the location of binding occurs in the major or in the minor groove of DNA.

Sperm DNA Condensation

A sperm cell contains half of the genetic information required for the creation of a normal embryo. During spermiogenesis the histones of the stem cells are partially replaced by transition proteins, which are then displaced by the protamines (1, 7, 8). This process is accompanied with a major change in the cell morphology and a great volume condensation (1, 9). The packaging of the DNA with the protamines in the sperm cell nucleus is probably different from the nucleosomal packaging of the histones in a stem cell, since the nuclear volume of a sperm cell is 50-fold smaller (2, 10). Due to the extreme compaction of DNA and protamines in the sperm head, DNA damage repair and transcription can not be performed (1). The DNA in the sperm cell is found to be biochemically inert and genetically inactive compared to the chromatin in a stem cell (2, 11).

Protamine

Protamines are small basic proteins, which are unique to the sperm cells. Even though there is not much conservation in the protamine sequence between various species (fish, birds and mammals), they have few common properties. All protamines contain a few arginine clusters and small amounts (10 to 25%) of non-arginine residues (1).

Fish protamine comprises a sequence of about 30 amino acids. There are usually more than one type of protamine found in each species (e.g., trout has six types). All of the fish protamine examined contain four long arginine stretches (4 to 6 residues) divided by nonpolar amino acids, such as proline, valine and glycine (1). The fish protamine resembles the central region of a mammalian protamine and was extensively used for the

investigation of the DNA-protamine complex formation (3, 4, 12).

There are two main types of protamine in mammals, protamine P1 and protamine P2. The sperm cells of some mammals, such as the bull, the ram and the rabbit, contain only one type of protamine (P1), and some of them, such as the mouse and the hamster, contain both the types. The human sperm was found to contain four types of protamines, out of which three are versions of the protamine P2 and one is of the P1 type (11, 13).

Protamine P1 has been found in all mammalian sperm cells investigated (13). It is a very small basic protein, which comprises 50 amino acids. In all P1 protamines there is a central polyarginine domain, which is divided into three clusters of arginine residues, separated by one to two non-arginine residues (14). Because of the large number of arginines, protamine P1 is very positively charged at neutral pH, causing the neutralization of the DNA's negative charge. The amino terminal sequence has remained very much conserved throughout evolution. The first six amino acids in all P1 protamines examined are identical (13). The importance of this sequence is yet unknown, but its conservation of sequence indicates that it has an important role, which may be in determining the direction of disulfide bonds in the formation of the protamines' secondary structure (14).

Protamine P2 is rich in histidine residues and is longer than protamine P1. A comparison between P2 protamines from various species revealed them to be very different from each other. There was not any apparent conservation of the amino acid sequence, and protamine P2 lengths varied between species (13).

The bull is an example of a mammalian organism whose sperm contains only one type of protamine, P1. Even though it does have a gene for protamine P2, this gene is not

expressed (15). For this reason, the bull protamine was selected as the model protamine for the examination of the DNA-protamine complex. Bull protamine P1 includes seven cysteines (7, 13), which can form disulfide bonds. It is known that there are two intramolecular bonds in each protamine and up to three intermolecular cross-links can be formed with other protamines (13). From protein-folding and DNA-binding experiments it was concluded that the amino and the carboxy terminals of the protamine are folded towards the center of the molecule (a staple shape), but it is unclear, whether they are directly involved in the formation of the DNA-protamine complex (11). **Figure 1** presents the shape of a protamine P1 molecule. Considering the protamine's staple-like shape, the central arginine region has been proposed to be in an extended formation in order to bind to the DNA molecule (7, 13).

DNA

DNA macromolecules are biopolymers containing thousands to millions of nucleotides. The monomeric units are four nucleotides containing one of four bases adenine (A), thymine (T), guanine (G), and cytosine (C), all of which are bound to a deoxyribose and connected by a phosphodiester backbone. The DNA found in living systems appears as a right-hand helix containing two strands, which are connected by hydrogen bonds between the bases. The shape of the double helix is determined by the sequence of the strands and the stacking of the bases above one other (16).

There are a number of different known forms of double stranded DNA, mainly A, B, and Z forms (16, 17). The A-form DNA is characteristic of the RNA double helix and

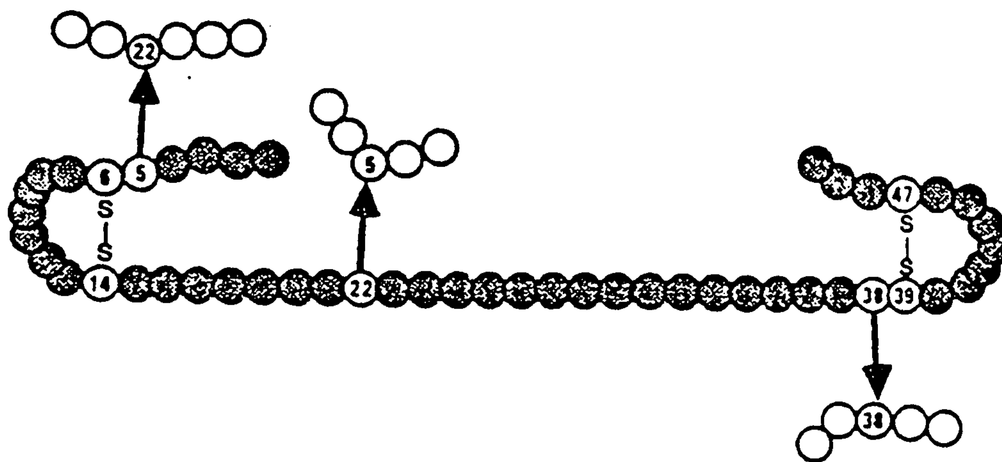
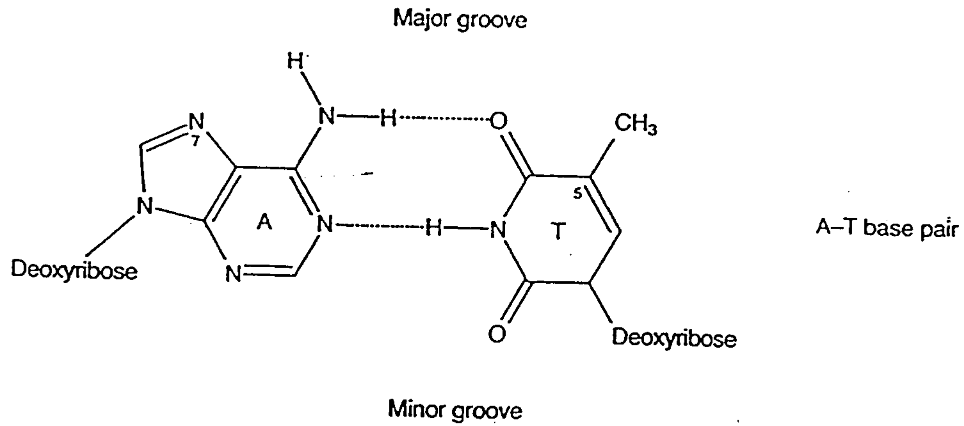


Figure 1: A diagram of the protamine molecule. The cysteine residues are indicated by numbers, and the inter- and intra-molecular disulfide bonds are shown by S-S or by bold arrows (13). (Reproduced with permission from Balhorn, 1989, Springer-Varlag.)

of the hybrid of DNA and RNA (during the transcription process, for example). The Z-form DNA is a left-hand helix in a long and thin structure, and was reported to be involved in the removal of basic proteins to allow supercoiling (16). The B-form DNA is the predominant form of DNA found in the nuclei. It has been proposed that the DNA in the sperm cell is in this form (2, 10) or in a slightly modified B form (3, 4, 18). The B-form DNA is a right-hand-turn helix, and the angle between the bases and the helix axis is dependent upon the specific DNA sequence; however, the general bases conformation is perpendicular to the helix axis (17, 19). Structurally, the B-form DNA is characterized by a wide major groove and a narrow minor groove (16, 17).

The DNA grooves can be distinguished by their width and depth and also by the functional groups present in each of them. **Figure 2** presents the bases and their functional groups with respect to the grooves. The major groove is characterized by the carbonyl of G, the amine of C, the amine of A, and the carbonyl and methyl of T, and the ring nitrogen, N-7, in A and G. The minor groove contains the amine of G, the carbonyl of C, the carbonyl of T, and the ring nitrogen N-3 of A (16).

When double stranded DNA is denatured either by heat or by an alkali, the hydrogen bonds break, the strands are separated and two single stranded DNA in a random coil shape are obtained (16, 20). The concentration of DNA in a double helix form can be measured using UV absorbance (20). It is known that nucleic acids absorb UV light in the 260 nm region (20, 21, 22). At neutral pH, one absorbance unit corresponds to 50 μg/mL of double stranded DNA, with an extinction coefficient of 20 cm^2/mg. When the double stranded DNA is denatured, the absorbency at 260 nm increases, since the bases



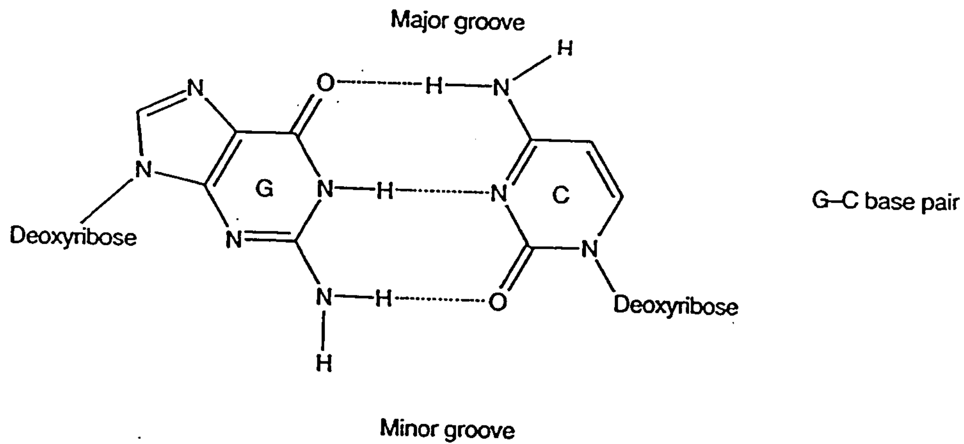


Figure 2: "Geometry of A-T and G-C base pairs and their orientation relative to the major and the minor grooves of DNA" (19). (Reproduced with permission from Travers, 1993, copyright 1993, St Edmundsbury Press.)

become unstacked (21, 23). For single stranded DNA, which is obtained from the denaturation of double stranded DNA, one absorbance unit at 260 nm corresponds to a concentration of 40 μg/mL, yielding an extinction coefficient of 25 cm^2/mg (21, 23). The increase in absorbency with the transition from double stranded to single stranded DNA is known as the hyperchromic effect (16). This hyperchromicity is used to determine the DNA state and to verify its structure.

Footprinting Methods

There are a variety of complexes of DNA and proteins involved in almost all of the activities in the living cell. Proteins, which are sequence specific, can recognize a particular sequence in the DNA chain and then bind to it at that site or at an adjacent site. The specific sequence can be as short as four bases. Another class of proteins are structural proteins, which can recognize structural features in the DNA molecule, such as the width or the depth of a groove or a special turn, and bind to it. These are the non-sequence-specific binding proteins and the protamines are considered to belong to this class (24, 25).

DNA and proteins are both macromolecules; however, the binding site of a protein on a DNA molecule can be very short in length. Some proteins are able to recognize a specific sequence of four to seven bases in a genome of about 10^9 base pairs and to form the complex by binding to this precise sequence. The sequence specific DNA-protein interactions are predominantly through hydrogen bonds between the protein and the individual bases (19). Since the structure of these complexes involves very large molecules

and very high binding specificity, these systems have been successfully investigated using biochemical methods such as various footprinting techniques.

DNA footprinting experiments have been used to generate binding site information about a number of DNA-protein complexes. This method enables the detection of a specific binding site or the determination of the DNA length that is accompanied with the protein binding. The first experiments were conducted with an enzyme, pancreatic DNase I, which provided the basis for a model of nucleosome structure (5). The nucleosomal histone proteins protect one side of the DNA, enabling the DNase I to access and thus cleave the other unprotected side. The possibility of protection of the DNA by the protein at the binding site is the essence of the footprinting technique.

Galas and Schmitz (26) were the first to publish a description of a footprinting experiment using DNase I and the sequence-specific DNA binding protein, *lac* repressor. The 5'-end-labeled DNA-*lac* repressor complexes were formed and were subjected to partial digestion by DNase I. The DNase I was used to cut the phosphodiester bond leaving a hydroxyl group on the 3' end and a phosphate group on the 5' end, hence cleaving the DNA molecule. The location of the binding site is determined relative to the 5' end and the length of the protected region can be determined by performing the experiment with both the strands. Denaturation and electrophoresis of the DNA fragments showed that only one region of the DNA remained uncleaved, whereas the rest of the sequence was cleaved randomly at many sites. Their conclusion was that the *lac* repressor protects this binding site from DNase I, not allowing its cleavage. This protected sequence

is the binding site of the *lac* repressor on the DNA molecule and this is its footprint.

Footprinting experiments have been used widely to examine complexes of various proteins and DNA oligomers. The footprinting method actually comprises two main complementary approaches, the protection technique and the interference technique. The protection technique is one in which the DNA-protein complex is first formed and then a chemical or an enzymatic reaction is introduced, which ultimately produces the cleavage of the DNA in the unprotected regions. The interference technique, on the other hand, monitors the ability of the protein and the DNA to form a complex after a chemical modification of the DNA either in its sequence, or in the backbone, or in the base(s). In both techniques, the DNA remains intact throughout the process. In the protection technique, when the reaction is completed, DNA isolated from complexes is denatured and electrophoresed in order to separate the fragments and to determine the binding site (5). When an interference experiment is conducted, electrophoresis of the reaction mixture causes the separation of the complexes and the free DNA and then isolation and a second electrophoresis enables the determination of the binding site.

Enzymatic protection experiments with DNase I are performed to discover binding sites for sequence specific proteins on the DNA. Control experiments with DNase I and free DNA indicated that DNase I has almost no sequence preference for binding and cleavage of free DNA (5). This enzyme is very efficient in cleaving the unprotected nucleotides and not damaging the protected ones. (The DNase I does not cleave any covered nucleotides.) This technique provides information on the length of the DNA-protein interaction site and on the uncovered sites to which other proteins may bind. It is

important, however, to understand the method's limitation, where one may mistakenly consider the entity of the covered domain as the actual binding site, while in reality the binding site may be shorter (5, 16).

Another enzymatic method of footprinting utilizes exonuclease III. This enzyme cleaves the DNA from the 3' end, nucleotide after nucleotide, up to the edge of the protein protected site. The use of exonuclease III enables only the detection of the protected site end, which, as in the DNase I method, is not always identical to the edge of the actual binding site. This method does not provide any other information about the complex or the binding site, but it is very accurate, although somewhat limited (5).

Protection and interference experiments can be based on chemical reactions with small molecules as well. Dimethyl sulfate is used for methylation of bases, ethylnitrosourea for ethylation of phosphodiester backbone, and hydroxyl radical for direct cleavage of phosphodiester backbone to show solvent accessibility. The methylation protection technique, using dimethyl sulfate (DMS), is applied in this research. A detailed explanation of this method as well as an overview of other applicable footprinting techniques will be presented in the following paragraphs.

Dimethyl sulfate (DMS) is a reagent used for the methylation of certain bases in the DNA grooves. In the B-form DNA, DMS methylates the N-7 of guanine in the major groove and the N-3 of adenine in the minor groove (5, 6). This methylation does not damage the DNA in terms of cleavages and nicks. The DMS methylation reaction is more efficient on guanine in the major groove and is thus used widely to determine contact or binding to this groove. The methylation modification can be used as a protection

experiment (e.g., methylation of the bases which are not protected by the protein) or as an interference experiment (e.g., methylation of the DNA and then subjecting it to the protein to check for binding). At the end of the experiment, the methylated DNA is reacted under basic conditions at high temperature, the base ring is eliminated and the backbone is cleaved at the apurinic site (5), thus enabling detection of methylation sites by standard DNA electrophoretic sequencing techniques.

A scheme for the methylation and the cleavage reactions is presented in **Figure 3**. Dimethyl sulfate methylates the N-7 of guanine causing a disruption in the electronic structure of this base and a generation of a positive charge in the ring. A base-catalyzed reaction opens the ring by breaking the bond between C-8 and N-9. Piperidine is then added to the reaction mixture and causes the displacement of the opened ring. The piperidine also causes a β-elimination reaction of both the phosphates from the sugar. This last reaction is the cleavage of the DNA at the modified site (6).

Ethylnitrosourea is a reagent used for ethylation of the phosphodiester backbone. This is an interference technique in which an ethyl group is bound to the phosphodiester backbone, causing, as a consequence, steric hindrance and the removal of the negative charge. The ethylation reaction does not exhibit any preference towards a specific base or groove in the DNA and thus much broader information can be obtained from this technique. The triester, which is formed by this ethyl substitution, is unstable under alkali and heat conditions, making it possible to cleave the phosphate backbone at the location of the interference. Ethylation interference experiments have been used to define binding sites of sequence-specific proteins.

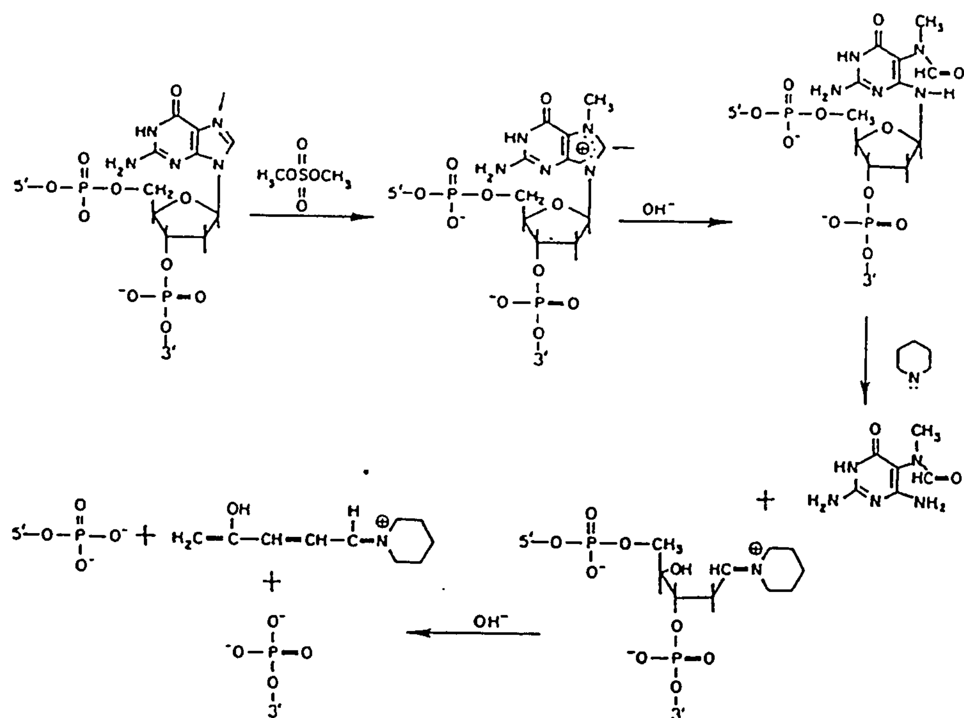


Figure 3: The reaction of dimethyl sulfate with guanine, the elimination of the base by piperidine and the break of the phosphodiester backbone (6). (Reproduced with permission from Maxam and Gilbert, 1980, Academic Press.)

The hydroxyl radical is a very small and reactive footprinting reagent, which marks solvent accessible positions on the DNA. The radical is neutral and thus approaches the phosphate backbone at any site which is not directly covered by the protein (5). In general, the hydroxyl radical attacks the DNA surface and has no sequence preference for its reaction and cleavage. The hydroxyl radical can be produced by a $[Fe(II)EDTA]^{-2}$ complex, when reacted with hydrogen peroxide (27, 28). This radical induces a displacement of a hydrogen from the deoxyribose and a chain cleavage reaction is initiated.

The investigations of several DNA-protein systems have been conducted using the footprinting techniques described above. The following paragraphs describe some of the experiments performed to determine the binding sites of proteins on DNA and to examine various binding interactions between them.

Siebenlist and Gilbert (29) determined the binding site of E. coli RNA polymerase on the A3 promoter of phage T7, by using ethylation interference, methylation protection and methylation interference experiments. The ethylation of the DNA backbone revealed that ethylation on both strands interfered with the polymerase-promoter binding; the methylation protection exhibited the exact binding site in terms of the sequence; and the domain covered by the polymerase when interacted with the promoter was determined by the methylation interference experiment.

Blackwell and colleagues (30) used a number of footprinting methods, such as methylation interference, ethylation interference and hydroxyl radical protection and interference experiments, in order to examine the Skn-1 protein binding site sequence, and

the location of this binding in terms of grooves. Skn-1 is a maternally expressed protein involved in correct cell specification during the early stages of the embryo's development. They reported that Skn-1 binds in the major and in the minor grooves along one face of the DNA helix to a specific sequence of five bases.

Hendrickson and Schleif (31) conducted binding interference experiments to investigate the binding site of E. coli AraC protein on DNA and its mode of binding. Three positions along the major groove were found to be bound by AraC protein, and these sites were reported to be located along one face of the DNA helix. The phosphodiester bond was proposed to play a role in the contact between the protein and the DNA.

Tullius and Dombroski (32) used the hydroxyl radical footprinting technique to determine contacts between DNA and two proteins, bacteriophage λ CI repressor and λ Cro protein. They proposed that both proteins attach to DNA on one face of the DNA helix. This data was not revealed by other footprinting methods, since they are base-dependent techniques (methylation) or modifications involving steric hindrance (ethylation). The hydroxyl radical has almost no sequence preference, and since it is small, only sites which are directly bound cannot be reached and cleaved.

All of the footprinting methods have been used to define specific sequences or precise binding sites of various proteins on DNA chains. Moreover, they have been used to determine binding to structural features like DNA grooves and sides. The footprinting methods are different but complementary to each other and together provide broad and accurate information about the system of interest. An important parameter that has to be considered when a DNA-protein system is examined using these methods is the

complexes' half-life. The half-life of the complexes should be long enough to enable the performance of chemical modifications while the system is intact. This is true for all of the footprinting methods, including methylation, which is the method used in this work.

DNA-Protamine Binding Experiments and Models

The DNA-protamine complexes form very compact structures, compared to the chromatin in the nucleus of a stem cell (10). A clear and proven explanation of this phenomenon has yet to be determined. It is proposed (7, 8) that when the protamines replace the histones, water molecules, which are associated with the DNA, are displaced allowing the volume reduction. But this is not sufficient to explain the consolidated structure. The condensation of DNA leads to the idea that the packaging of the chromatin in the sperm cell is different from the way the nucleosomes are packed (10). It may be that the protamines are somehow bound in the DNA grooves, utilizing the remaining volume (2, 7). The protamine binding sites on the double stranded DNA have been investigated since the 1970s. It is well accepted that protamines are bound to the DNA through hydrophobic and electrostatic interactions, however, the arrangement of the protamines is yet to be determined (7, 33).

The following reports are based on experiments conducted with fish sperm cells or isolated fish protamines. Mirzabekov et al. (12) utilized a methylation protection experiment with [^{3}H]dimethyl sulfate to investigate the binding site of protamine on DNA. The experiment was designed to measure the methylation rate on N-7 of guanine in the major groove and the rate on N-3 of adenine in the minor groove. In the in vitro

experiment, calf thymus DNA and purified salmon protamine were used. They proposed that the protamines are bound in the major groove of the DNA, since the ratio of m^7G/m^3A in DNA bound with protamine was decreased by 21% compared to free DNA. In addition, an in vivo experiment was conducted with intact fish sperm cells. The overall methylation ratio of m^7G/m^3A of the DNA in the cell was reduced by 14% compared to free DNA. The in vivo and the in vitro experiments led to the conclusion that the protamines preferentially bind to the DNA in the major groove. The minor groove was found to be open and exposed to methylation in the protamine bound DNA compared to the free DNA (12).

Suau and Subirana (3) conducted X-ray diffraction studies of the location of the protamine on DNA. Cuttlefish and squid protamines were purified and used with calf thymus or sea urchin DNA. It was reported that protamines considerably reduced the DNA hydration and the complex composition was not effected by humidity variations. Moreover, the DNA was found to be present in the B form at all humidity levels (0 to 92%). The DNA-protamine complex was determined to be packed in a hexagonal system with a single DNA molecule in each unit cell. The electronic density between the sugar and the phosphate backbone was higher in the DNA-protamine complex than in the free DNA. Suau and Subirana interpreted the higher electronic density as an indication that the protamines are bound in the minor groove. Two models were proposed for the DNA-protamine complex arrangement. In each model the protamine is bound in the minor groove. In the first model (**Figure 4a**), the projecting regions of the protamine fit in the neighboring major groove, even though there are not any connections between the DNA

molecules themselves. In the second model, a connection between the DNA helices by the protamines is proposed. One molecule of protamine is bound to several different DNA molecules, bridging them as shown in **Figure 4b**.

In the models of Suau and Subirana (3), although the protamine was proposed to be bound to the minor groove, the shape of this protein when bound to the DNA was not mentioned. Warrant and Kim (34) utilized yeast phenylalanine tRNA and salmon protamine as their model system, even though the tertiary structure of tRNA is different in shape from double stranded DNA. Circular dichroism (CD) and X-ray diffraction were used in their efforts to determine the protamine shape when bound to the nucleic acid and to determine the protamine binding site.

The CD of isolated protamines in a buffer solution showed a random coil shape. The tRNA and the protamine when placed together precipitated immediately, and therefore did not yield any CD results for the complexes formed. For the X-ray diffraction study, crystals of tRNA were prepared and then soaked in protamine solution to allow complex formation. The resulting electron density showed one peak, which corresponded to an α-helix segment of 9 to 10 amino acids. Since there is a total of 32 amino acids in the protamine, they proposed a structure of four such segments bound to the minor groove of the tRNA. In addition, the results revealed that the protamine binds to both the tRNA strands in the minor groove and with two more strands on two different neighboring helices. This arrangement stabilizes the double stranded tRNA helices, and provides a tight packaging form.

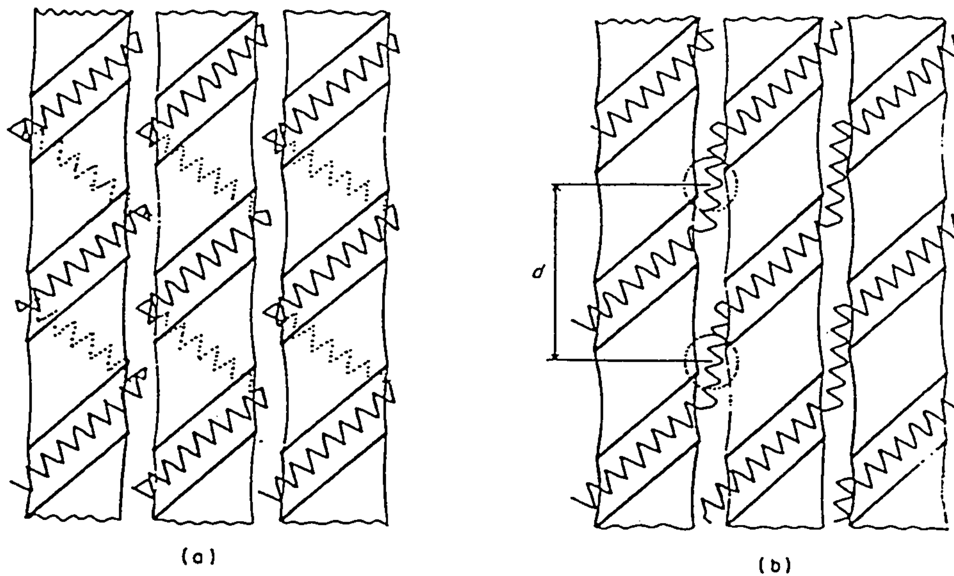


Figure 4: The DNA-protamine complex. The DNA is presented by heavy lines and the protamine is presented by the zigzag line. The protamine is bound to the minor groove. [a] "Protamines are wrapped around individual DNA molecules. The protruding ridge of protamine fits in the wide groove of neighboring DNA molecule." [b] "Regions which are rich in protamine appear between neighboring DNA molecules." (3). (Reproduced with permission from Suau and Subirana, 1977, Academic Press.)

The conclusions inferred from the results were that the protamines have an α-helix shape when bound to the tRNA, and that the protamines are bound in the minor groove of tRNA (and probably other nucleic acids). **Figure 5** presents the proposed protamine conformation when bound to the DNA. The X-ray results indicated that the protamine comprises four α-helix portions (8 to 9 amino acids in each) connected by flexible joints, which form a tight packaging of RNA or DNA, since one protamine binds to more than one helix.

The computer model presented by Warrant and Kim (34) in the same paper is based on the results obtained from the X-ray diffraction experiment. **Figure 6** presents a scheme of the binding model. The protamine was considered to exist as four α-helix segments linked together, which are able to connect neighboring double helices. In contrast to the experimental results and conclusions, the computer model suggested that the most probable binding site is actually the major groove, although the minor groove is also a possibility.

X-ray diffraction of salmon spermheads was used by Suwalsky and Traub (35) for the investigation of the DNA found in the sperm cell and the binding site of the protamine on the DNA. They reported that the orientation of the DNA fibers coincides with the B-form DNA, and proposed the packing of the spermhead to be in a hexagonal system with one DNA molecule in a unit cell. The X-ray pattern seemed to be in agreement with the binding of protamines to the minor groove of the DNA; however, an increase in the intermolecular distance with the increase in humidity Indicated binding to more than one

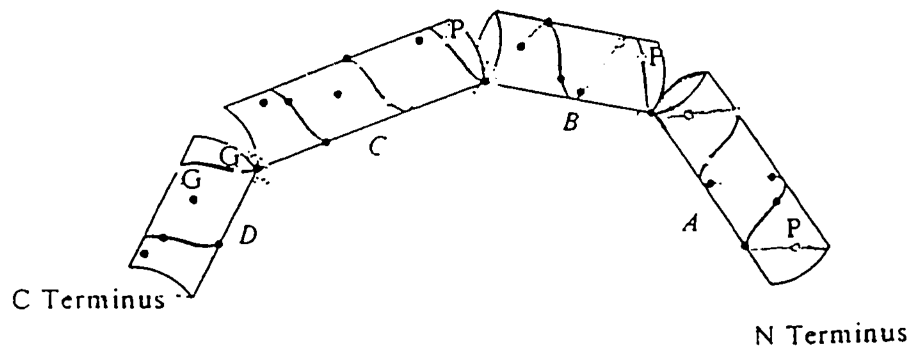


Figure 5: A schematic presentation of a fish protamine, comprises four α-helices, when bound to the DNA (34). (Reproduced with permission from Warrant and Kim, 1978, Macmillan Magazines Ltd.)

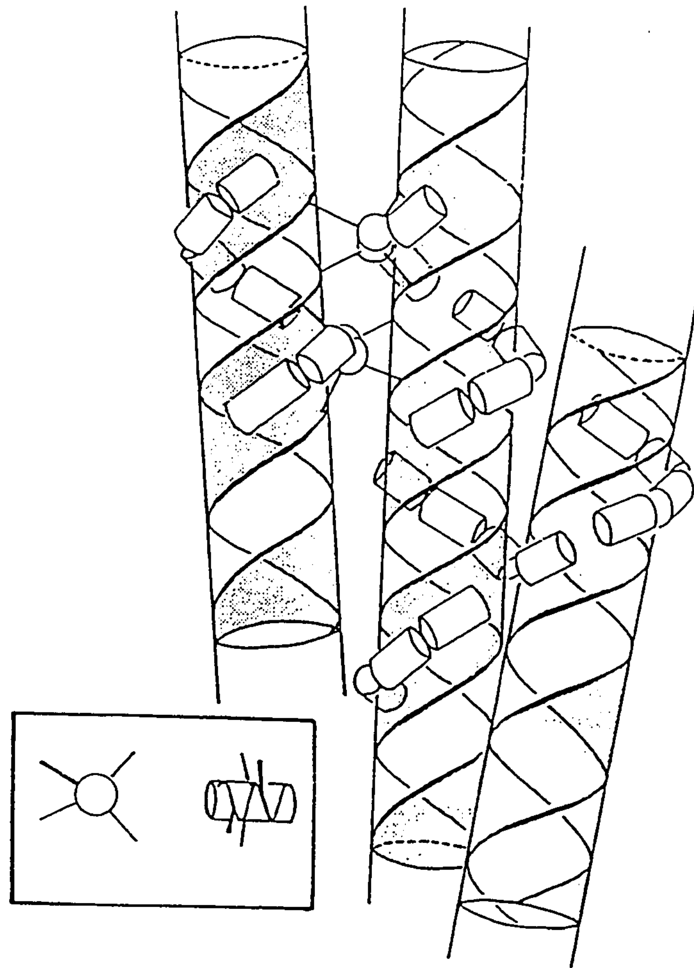


Figure 6: A computer model of DNA-protamine complex. The protamine is bound in a α-helix conformation to the major groove of DNA, and arginine residues connect the neighboring helices through the major and the minor grooves (34). (Reproduced with permission from Warrant and Kim, 1978, Macmillan Magazines Ltd.)

DNA helix.

Herskovits and Brahms (18) used infrared linear dichroism, UV circular dichroism and Raman spectroscopy to demonstrate that B-form DNA is slightly modified upon protamine binding. The angles formed between the oxygens and the phosphate (OPO) cause the phosphodiester backbone to be orientated differently from the B-form DNA. The protamines were concluded to be bound in one of the DNA grooves, without specifying in which of the two grooves.

X-ray diffraction studies were conducted by Fita et al. (4) for the determination of the binding site on B-form DNA by arginine, polyarginine, and various polypeptides containing stretches of arginines and nonpolar amino acids. They concluded that the unit cell dimensions are consistent with B-form DNA and that it is orthorhombic in packing. This type of packing means that the arginine residues of the protamines are probably bound in the major groove of the DNA, since only the major groove would be available. The nonpolar amino acids are assumed to be projected from the complex in order to allow maximum interaction of the arginines and DNA phosphates, and placed in the minor groove of a neighboring DNA helix.

The previous experiments and models have involved fish DNA-protamine interactions, which are predominately arginine clusters with occasional non-arginine spacer amino acids. The following DNA-protamine binding models deal with mammalian protamine. The central portion of the mammalian protamine, which contains the arginine residues, is thought to be bound to the DNA. The location of the C- and the N-termini of

mammalian protamine when bound to the DNA is unknown.

A model for the mammalian protamine-DNA binding was proposed by Balhorn (7) in 1982. In this model, the protamine is assumed to be in a random coil conformation in solution or in an extended shape when bound on the DNA. Only the central portion of the protamine (amino acid residues 16 to 36) is proposed to be bound to the DNA in its minor groove, since it was calculated that accommodation of the entire protamine molecule in the DNA groove requires more than twice the amount of DNA found in the sperm cell. One protamine central domain covers exactly one turn of the DNA helix, while the C and the N terminals are free to interact with other DNA molecules in the formation of the condensed structure. **Figure 7** presents a scheme of the binding model. The guanidinium groups of the arginine residues (positively charged) are proposed to be arranged in alternating directions, enabling the fitting of the protamine inside the groove and providing complete charge neutralization. The neutralization minimizes the repulsion between the DNA molecules, allowing the protamine's termini to bind in a major groove of an adjacent DNA molecule. This composition of the protamine and the DNA in a multiple DNA-bound complex results in a very tight package and furnishes an explanation for the condensation. This model is reported to be based upon infrared data and upon the calculation that a protamine with an α-helix conformation (34) would only cover half of the DNA grooves in a sperm cell. In addition, an α-helix shape would not fit in the grooves' width and thus could not neutralize the DNA's negative charge.

Side by side with the experimental research of the DNA- protamine complexes,

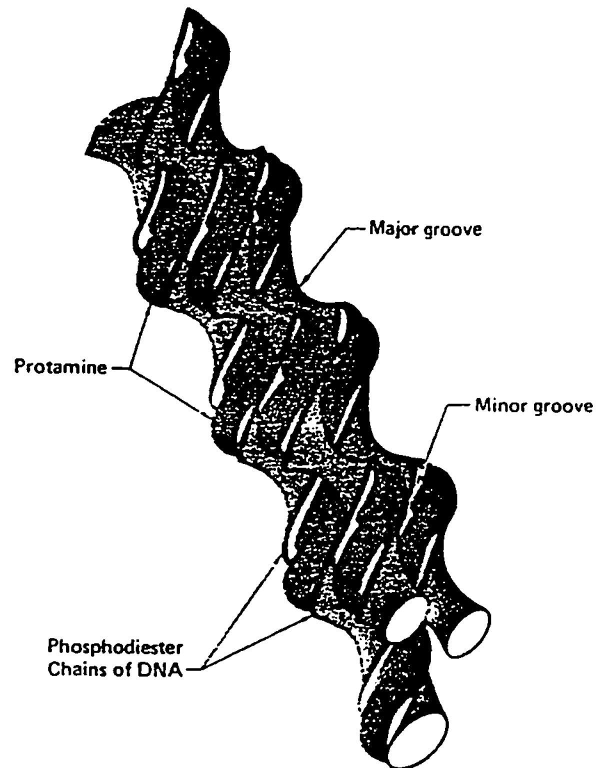


Figure 7: A mammalian protamine-DNA binding model. The protamine is bound to the minor groove and adjacent DNA helices are connected through the major groove (7). (Reproduced with permission from Balhorn, 1982, Rockefeller University Press.)

computer models describing the binding sites exist, based on known data and measurements. In 1992, Hud and co-workers (36) came up with a new computer model, which considered the protamine to have an extended or a 2_7a-ribbon shape and alternating directions of the guanidinium groups of each consecutive arginine. The protamine's shape or the polypeptide backbone's conformations were determined using infrared studies and Raman spectroscopy. The model also assumed that one protamine central region covers one turn of the DNA helix and that the DNA is in B form. Applying fish protamine with a 30 base pair DNA chain to the model, yielded the binding of the protamine in the major groove. According to the computer model, the protamine is placed along the major groove, where the arginine portions are forced to be in a "near planar" structure, and the non arginine residues (located between the arginine portions) contribute the ability to follow the turns of the DNA groove, working like hinges.

Many models representing the organization of the DNA-protamine complex have been proposed. The experimental models suggested that protamine is bound in the minor groove of DNA as a random coil or as an α-helix. The computer models and some experimental results have shown binding to the major groove with either an α-helix shape or an extended protein conformation.

DNA-Protamine Interaction and Kinetics

The DNA-protamine complex is insoluble; it aggregates and precipitates even at nM concentrations when formed in a free solution (Stacks, unpublished results). Another approach is required to provide complexes which would be separable, soluble, and not

aggregate. Bull P1 protamine, substituted on an activated thiol sepharose 4B resin and attached to it via a disulfide bond, has been used to examine DNA-protamine interactions (33). Due to the immobilization of the protamine, the aggregation of the complexes was avoided. DNA oligomers were labeled with [^{33}P-γ]ATP at the 5' end, in order to quantitate the free and the bound DNA.

When a highly substituted resin was used (252 pmol of protamine per 1 milligram resin), the experiments showed irreversible binding and almost no release of labeled DNA (33). It was unclear whether this was due to the complex being irreversibly bound or whether there were too many binding sites available, such that each dissociation resulted immediately in a binding to another site. The use of a highly concentrated NaCl solution (2 M) or inclusion of an excess of unlabeled DNA enabled a very rapid and efficient dissociation of the complexes. Less substituted resin (63 to 126 pmol P1 per 50 milligram resin) yielded reversible binding of complexes in the presence of a 0.1 M NaCl solution, and was used to gather kinetic data on the complex (33).

Kinetic studies conducted with the low substituted resin yielded a half-life of the DNA-protamine complex of approximately 117 minutes (33). The association rate constant was calculated to be $k_1 \cong 1.1 \times 10^6$ M^{-1} s^{-1} (33, 37) and the dissociation rate constant was found by this system to be $k_2 \cong 8.9 \times 10^{-5} \pm 4 \times 10^{-5}$ s^{-1}, therefore the dissociation constant was determined as $K_D \cong 8.1 \times 10^{-11} \pm 4.0 \times 10^{-11}$ M (33). The value of the dissociation constant revealed that the DNA-protamine complex is a very high affinity complex ($K_D < 10^{-8}$ M characterizes high affinity complexes) (37). The dissociation

constant was shown to be related to the oligonucleotide length. Rapid dissociation was reported to occur from the 10 base pair oligomer, while the dissociation from the 12 and the 14 base pair oligomers was similar. Additionally, the results were consistent with the involvement of electrostatic forces in the interaction between the DNA and the protamine in the formation of the complexes (33).

The Research Objective

When a low protamine substituted resin is used, the binding of protamine and DNA is reversible as indicated by the dissociation of the complex upon dilution. Moreover, the dissociation can occur in mild conditions (e.g., low NaCl concentration). Since the half life of the complex is approximately 117 minutes, the stability of the complexes allows a biochemical analysis of its binding.

The main objective of this work is the determination of the binding location of protamine P1 on a 12 base pair oligonucleotide (i.e., binding in the major groove, the minor groove, or outside of the grooves). A methylation protection experiment was designed in order to determine in which of the DNA grooves the protamine binds.

A double stranded B-form DNA is required for the investigation of the protamine binding site in the DNA-protamine complex. The stability of the double stranded DNA is crucial for the complex to maintain its structure and to provide a reliable model system. The characterization of the stability of double stranded DNA, comprising 10 to 22 base pair oligomers, is the first objective of this work.

A synthetic 12 base pair DNA oligomer and a low substituted resin (0.63 pmol per 50 milligram resin) was used to form the reversibly bound DNA-protamine complex. Methylation with dimethyl sulfate on the exposed DNA sites, either in the major groove or in the minor groove, and a subsequent cleavage, produced DNA fragments to be separated by electrophoresis. The resulting electrophoretic pattern would be used to determine the location of the protamine binding site. The comparison of the bound DNA cleavage pattern with the bands produced by the cleavage of the free DNA would indicate the binding location.

The biochemical significance of this research is to provide more data about the DNA-protamine system, to assist the development of a more accurate and reliable model system. The numerous models proposed are quite different from each other in terms of the grooves the protamines lie in and in terms of the protamine conformation when bound. Therefore, any data regarding the exact binding location of protamines on the DNA may enable the construction of a faithful model.

Materials and Methods

Buffers

The HEPES buffer used for the preparation of most of the solutions comprises HEPES-acid, which is N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid], and its sodium salt. A pH 7.4 solution of 50 mM HEPES was obtained by mixing a 50 mM HEPES-acid and a 50 mM HEPES-Na salt solution. The HEPES salt and the acid were purchased from Sigma and Aldrich.

The NaCl solutions (0.1 M and 2 M) were prepared volumetrically in a 50 mM HEPES buffer, pH 7.4. The sodium acetate (NaOAc), the magnesium acetate ($Mg(OAc)_2$), and the guanidine hydrochloride solutions were prepared volumetrically in water.

The T4 polynucleotide kinase was dissolved in a buffer containing: 50 mM Tris HCl pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, 100 mM NaCl, 0.1% Triton X 100, and 50% glycerol (obtained from Kodak International Biotechnologies). Buffer C, obtained from Kodak International Biotechnologies, contained 100 mM $MgCl_2$, 500 mM Tris HCl, 50 mM dithiothreitol, 10 mM EDTA, and 1 mM spermidine. The Quickspin™ columns, obtained from Boehringer Manheim, contained Sephadex G-25, fine, in 10 mM Tris HCl, 1 mM EDTA, and 0.1 M NaCl.

Materials

HPLC purified synthetic DNA oligomers of various sizes (7, 10, 12, 14, and 22

base pair oligomers) and HPLC purified bull P1 protamine were obtained from Dr. Rod Balhorn (Lawrence Livermore National Laboratory, LLNL). The radioactive material was purchased as [^{33}P-γ]ATP from NEN Research Products (DUPONT). T4 polynucleotide kinase [EC 2.7.1.78] was purchased from Kodak International Biotechnologies. Cytoscint solution was purchased from ICN Biomedical Inc. The 1000 MWCO (Molecular Weight Cut Off) dialysis tubing was purchased from Spectrapor. The nitrocellulose filter papers type HA, 0.45 μm pore size, were purchased from Millipore Corporation. Activated thiol Sepharose 4B resin, dimethyl sulfate (DMS), acrylamide, bisacryamide, urea, EDTA, ammonium persulfate, N, N, N', N',-Tetramethylethylenediamine (TEMED), Fuji GBX films (5x7 and 8x10 in.), GBX developer and GBX fixer were all purchased from Sigma Chemical Company. Piperidine was purchased from Aldrich Chemical Company, Inc. and formamide from BRL Life Technologies, Inc.

DNA Oligomers

Deblocked single stranded DNA as well as the double stranded DNA were purified by reversed phase high performance liquid chromatography (HPLC) in the laboratory of Dr. Balhorn. The sequence of these oligomers was designed to produce the B form of double stranded DNA. The concentration of the DNA was determined by UV spectroscopy using an extinction coefficient of 20 cm^2/mg at 260 nm. The sequences of the synthetic oligomers are presented in **Table 1**.

Oligomer size	**Sequence**
7 base pair	5'- OH CCG TAG G - 3' 3' - GGC ATC C OH - 5'
10 base pair (60% GC[1])	5'- OH GCA TGT ACC G - 3' 3' - CGT ACA TGG C OH - 5'
10 base pair (30% GC)	5'- OH GAA TGT ACA A - 3' 3' - CTT ACA TGT T OH - 5'
12 base pair	5'- OH TCG AAT GTA CAA - 3' 3' - AGC TTA CAT GTT OH - 5'
14 base pair	5'- OH GTT CGA ATG TAC AA - 3' 3' - CAA GCT TAC ATG TT OH - 5'
22 base pair	5'- OH TGC AGT TCG TTC GAA TGT ACA A- 3' 3'- ACG TCA AGC AAG CTT ACA TGT T OH - 5'

[1] GC rich at the ends for the stability of the double stranded DNA

Table 1: The sequences of the deblocked synthetic DNAs.

The Stability of the Double Stranded DNA Oligomers

The various DNA oligomers were diluted with 50 mM HEPES, pH 7.4, to a concentration of approximately 50 μg/mL and 1-mL samples were prepared. Denaturation of the DNA from double stranded to single stranded form was conducted in a 0.2 M NaOH solution (by the addition of 66 μL of 3 M NaOH solution) to the DNA sample. The complete denaturation was achieved after 5 minutes of incubation in the NaOH solution. A HP 8452 diode array UV spectrophotometer was used to measure the absorbance of the DNA samples at 260 nm. A blank of 50 mM HEPES, pH 7.4, was used for neutral pH absorbance measurements. A blank of 0.2 M NaOH in 50 mM HEPES buffer solution was measured for the denatured samples.

The Beer-Lambert equation relates the absorbency, the extinction coefficient, the concentration, and the length of the pathway:

$$A_{\lambda} = \varepsilon * l * c, \qquad \text{(equation 1)}$$

where A_{λ} is the absorbency units at a particular wavelength, λ, ε is the extinction coefficient, l is a path length, and c represents the concentration.

The stability of the DNA in the double stranded form was determined from the ratio between the absorbance at 260 nm of the DNA in alkaline and neutral pH, after including a correction for dilution:

$$A_{260,\,alkali}/A_{260,\,neutral} = [\varepsilon_{alkali}/\varepsilon_{neutral}] * [l/l] * [c_{alkali}/c_{neutral}] \qquad \text{(equation 2)}$$

$$c_{alkali} = c_{neutral}/1.066 \qquad \text{(equation 3)}$$

$$[A_{260,\,alkali}/A_{260,\,neutral}] * 1.066 = \varepsilon_{alkali}/\varepsilon_{neutral} \;. \qquad \text{(equation 4)}$$

Because of the hyperchromic effect, the extinction coefficient of the denatured DNA (ε_{alkali}) should be higher than that of the native DNA ($\varepsilon_{neutral}$). An extinction coefficient ratio [$\varepsilon_{alkali}/\varepsilon_{neutral}$] greater than one would indicate a transition from double stranded to single stranded DNA, and thus, stability in the double stranded form. A ratio less than or equal to one would indicate no transition and thus, instability of the double stranded DNA.

5' DNA End-Labeling

The double stranded DNA oligomers were labeled enzymatically at the 5' end with [^{33}P-γ]ATP and T4 polynucleotide kinase. The [^{33}P-γ]ATP contained 4500 Ci/mmol or 0.25 mCi/0.025 mL in 10 mM tricine at the date stated by the manufacturer. The labeling process was conducted according to the technique suggested by Lillehaug et al. (38), which was improved by Maxam and Gilbert (6).

The actual DNA labeling was performed in an Eppendorf tube. DNA (100 pmol DNA 5' ends) in 50 mM HEPES buffer was mixed with 1 μL of T4 kinase (8 to 10 units), 2 μL of buffer C, 5 μL of [^{33}P-γ]ATP (50 μCi/100 pmol DNA ends) and enough deionized water to bring the mixture to a final volume of 20 μL. The DNA reaction was placed in a water bath at 37 °C for 45 minutes, and then chilled on ice for 30 minutes.

The buffer in the Quickspin™ columns was exchanged to a 50 mM HEPES buffer, pH 7.4, by following the protocol of the manufacturer. Quickspin™ columns were used to provide the separation of the labeled DNA from the free [^{33}P] ATP. After addition of 30 μL of 50 mM HEPES to the 20 μL reaction mixture, 2 μL were removed to a scintillation

vial in order to quantitate the radioactive material present in the tube. The rest of the mixture was loaded onto the buffer exchanged Quickspin™ columns and centrifugation at 2000 g for 90 seconds. The labeled DNA was gathered into a new tube and 2 μL were removed to determine the extent of radioactive phosphate incorporation into the DNA. Cytoscint solution (5 mL) was added to the vials and the quantitation of the radioactivity was performed by a Packard Tri-Carb Liquid Scintillation Analyzer 1900CA model. The windows were set for phosphorous 32: Region A = 5.0-1700; Region B = 50.0-1700; Region C = 0-0; 2σ% was at 0 for all regions, and background correction was not applied. The incorporation calculation is based upon the assumptions that all of the DNA loaded on the column is recovered, and that there is similar quenching of [^{33}P] in the pre- and the post-centrifugation samples. The DNA used in these studies was labeled with initial specific activity of 0.125 to 0.275 μCi/pmol DNA ends.

Protamine

HPLC purified bull P1 protamine was isolated in the laboratory of Dr. Balhorn (39) and resulted in a single band on acid urea gel electrophoresis. The concentration of the bull P1 protamine was determined by UV absorbance measurements. The molar extinction coefficient of protamine in 10 mM HCl is 38,500 M^{-1} cm^{-1} at a wavelength of 230 nm (33).

Upon storage in 10 mM HCl, aggregation of the protamine occurs over time. Reduction of the bull protamine was carried out during an overnight incubation with 7 mg

of lyophilized protamine, 54 mg of dithiothreitol (DTT) and 2 mL of 6 M guanidine HCl in 10 mM Tris, pH 8 (Balhorn, personal communication). The reduced protamine was transferred to a 1000 MWCO Spectrapor dialysis bag. The sample was exhaustively dialyzed against 10 mM HCl. The diffusate was used as the blank for the absorbance determination of the protamine concentration.

The Resin

The Sepharose 4B resin is a cyanogen bromide activated porous resin with a glutathione 2-pyridyl disulfide group attached to it (Pharmacia). The protamine binds to the resin through disulfide bonds and thus releases 2-thiopyridine upon binding. The disulfide bond is formed between one of the cysteines in the protamine sequence and the thiol group on the resin. The amount of the 2-thiopyridine released upon binding is not sufficient for the detection of low protamine substituted resin by absorbency. The extent of substitution is actually determined by reversible binding of DNA to the protamine.

Protamine Substitution on the Resin

Fifty milligrams of Sepharose 4B resin was swelled in 16 mL of 50 mM HEPES, pH 7.4, for an hour at room temperature. The resin was pelleted by centrifugation for 30 seconds at 17,000 g. The supernatant was discarded and the resin was transferred to an Eppendorf tube. A volume of 800 μL of 50 mM HEPES, pH 7.4, was added to the resin. Protamine (63 or 126 pmol) was resuspended in a 10 mM HCl solution, yielding a final volume of 200 μL. The protamine solution was added to the resin for incubation at room

temperature for one hour on a shaker.

The substituted resin was then centrifuged for 20 seconds at 17,000 g and the supernatant was discarded. To remove non-covalently bound protamine, 0.5 mL of 5 M guanidine HCl in 50 mM HEPES, pH 7.4, was added to the resin and incubated for 5 minutes. After centrifugation, the separated resin was washed twice with 0.5 mL of 0.1 M NaCl in 50 mM HEPES, pH 7.4. After the final centrifugation, the substituted resin was brought to a volume of 1 mL with 0.1 M NaCl in 50 mM HEPES, pH 7.4.

A control resin was treated identically; however, a solution of 200 μL 10 mM HCl was added to the swelled resin instead of the protamine solution.

Dissociation

One fifth of the volume of the resuspended substituted resin (approximately 10 mg in 200 μL) was added to an Eppendorf tube with 10 μL of [^{33}P]-labeled DNA (specific activity of 0.25 to 0.55 μCi/pmol DNA ends) and incubated for one hour. The tube was placed in a shaker, in order to obtain a uniform exposure of DNA to the substituted resin. After the incubation period, samples of 10 μL were removed into a series of Eppendorf tubes, which contained 990 μL of the appropriate dissociation solution. The dissociation time varied from 0 to 1 hour (in intervals of 15 minutes), and 2, 3, 4, and 24 hours. At the appropriate times, the samples were centrifuged at 17,000 g for 20 seconds and 100 μL of the supernatant was removed into a vial with 5 mL of scintillation liquid (Cytoscint) to determine the amount of free DNA. The rest of the material in the tube was mixed and

filtered immediately. The nitrocellulose filters, were used to capture the resin-bound DNA-protamine complexes. The filters were prewetted with deionized water. The filter was added to a vial containing 5 mL scintillation liquid to quantitate the bound DNA. Non-specific DNA binding to the resin was determined by the use of unsubstituted resin in the same procedures, and non-specific binding to the filter was determined by the use of labeled DNA mixed in the appropriate dissociation solution applied directly on the filter.

A number of salt solutions were examined to determine the extent of dissociation of the complexes. The standard solution of 0.1 M NaCl in 50 mM HEPES, pH 7.4, was used to determine the percentage of initially bound DNA to the protamine. Other dissociation solutions used were 2 M NaCl in 50 mM HEPES buffer at either pH 7.4 or pH 8.3, 3 M NaOAc pH 8.3, 3 M NaOAc in 50 mM HEPES, pH 7.4, and 3 M $Mg(OAc)_2$ in water pH 8.1.

Methylation and Cleavage of DNA

Chemical methylation and cleavage methods were conducted according to the method proposed by Maxam and Gilbert (6) with slight modifications. In an Eppendorf tube, 50 μL of substituted resin (0.63 pmol per 50 mg resin) and 10 μL of labeled DNA were mixed. The mixture was then incubated in a shaker for 1 hour at room temperature to form the DNA-protamine complexes. Then 1 μL of DMS was added, and the mixture was incubated for 10 to 15 minutes at 20 °C. Dilution of the mixture in 940 μL of 0.1 M NaCl in 50 mM HEPES buffer decreased the methylation reaction by causing the dilution

of the methylation agent, DMS. The subsequent steps up to the overnight drying in the desiccator were conducted in the cold room at 0 to 4 °C. The mixture was centrifuged for 15 seconds at 13,000 g. The supernatant (containing the free DNA and excess DMS) was removed and kept aside. To the resin left in the Eppendorf tube (~ 50 μL), an equal volume of stop solution was added. The stop solution is added in order to stop the methylation reaction completely, as well as to dissociate the DNA from the complex and to obtain the free, methylated, labeled DNA, which had been bound to the protamine during the methylation process. The stop solution comprises 4 M NaCl, 1 M β-mercaptoethanol and 100 μg/mL of sheared calf thymus DNA. The resin was mixed with the stop solution and then centrifuged for 15 seconds at 13,000 g. The supernatant, which contains the previously bound DNA, was removed to a new Eppendorf tube, and the remaining resin was filtered after the addition of 0.5 mL of 0.1 M NaCl in 50 mM HEPES, pH 7.4. The filtration of the resin was conducted in order to verify the amount of DNA still bound to the protamine substituted resin after the dissociation process.

For the precipitation of the methylated DNA, an additional 50 μL of 4M NaCl stop solution (to improve precipitation), 150 μL of 0.5 M NaOAc (at 4 °C) and 750 μL of 95% ethanol (at 4 °C) were added. The DNA solution was centrifuged for 30 minutes at 13,000 g. The supernatant was removed into a vial containing a scintillation liquid to count the amount of DNA lost in this wash, as was done with each subsequent precipitation and wash step. To the DNA left in the tube, 250 μL of 0.3 M NaOAc (at 4 °C) and 750 μL of 95% ethanol (at 4 °C) were added in order to precipitate and to wash

the methylated DNA. The sample was centrifuged for 15 minutes at 13,000 g. This last wash was repeated twice, and the third wash was performed with only 1 mL of 95% ethanol. The DNA-ethanol mixture was centrifuged for 15 minutes, the supernatant was removed and the DNA left in the Eppendorf tube was then dried overnight in a desiccator connected to the house vacuum.

To the dried, methylated DNA, 100 μL of 1 M piperidine (freshly diluted) was added. The DNA was dissolved in the solution and a two-second centrifugation provided for the collection of the material at the bottom of the tube for the initiation of the DNA cleavage process. The Eppendorf tube was capped and sealed with Teflon tape, and the tube was heated in a water bath at 90 °C for 30 minutes. (The cleavage process occurs at this stage.) The tube was removed to cool for 5 minutes at room temperature and then centrifuged for 30 seconds at 13,000 g. The sample was frozen and lyophilized. After the lyophilization, 10 μL of water was added, the DNA dissolved, centrifuged for 30 seconds, and then frozen and lyophilized. The wash with 10 μL of water was conducted twice, and at the end of the procedure only fragments of DNA were expected to remain in the tube.

In order to separate the DNA fragments resulting from the methylation and cleavage reaction by electrophoresis, 6 μL of loading buffer was added. The loading buffer comprises 80% formamide, 10 mM NaOH, 1 mM EDTA and 0.1% (w/v) xylene cyanol. The sample was then centrifuged for 5 seconds and heated to 90 °C for 1 minute, in order to completely denature the DNA. After heating, the sample was immersed in an ice-water bath for fast chilling to prevent reannealing of the DNA. One microliter of the DNA

loading buffer mixture was removed to a vial containing a scintillation liquid for quantitation. The rest of the DNA was then loaded on an acrylamide-urea gel and the Eppendorf tube was washed with scintillation liquid, then counted to find out the amount of DNA lost.

Two control samples were used for this experiment. One control was performed to verify the methylation and the cleavage techniques. The [^{33}P]-labeled 12 base pair DNA in 0.1 M NaCl in 50 mM HEPES, pH 7.4, was processed according to the protocol proposed by Maxam and Gilbert (6), and provided the free DNA cleavage pattern.

The other control was the free DNA which was removed as a supernatant after the methylation of the DNA-protamine complexes. This free DNA was methylated at the same time as the DNA-protamine complex, however, with no protamine protection. Approximately 950 μL of supernatant were removed and 95 μL of it were counted to give an indication of the total free DNA amount and from this the bound DNA amount. Another 50 μL of the supernatant was removed into an Eppendorf tube and the methylation and cleavage process continued according to the protocol described above.

The efficiency of the stop solution in terminating the methylation reaction was tested in an experiment based on the protocol proposed by Maxam and Gilbert (6). For this, three samples were prepared. The immediate reaction of the stop solution was examined in the first sample, where 50 μL of 0.1 M NaCl in 50 mM HEPES, pH 7.4, was incubated with 1 μL of the methylation reagent (DMS) for 10 to 15 minutes. Next, the stop solution (50 μL) was added and 10 μL of [^{33}P]-labeled 12 base pair DNA was added

immediately after. The effectiveness of the stop solution after a time period of 5 minutes was examined in the second sample, where 50 μL 0.1 M NaCl in 50 mM HEPES, pH 7.4, was incubated with 1 μL of the methylation reagent (DMS) for 10 to 15 minutes. The stop solution was added (50 μL) and 5 minutes later 10 μL of [^{33}P]-labeled 12 base pair DNA was added to the mixture. For comparison, a third sample was processed according the protocol, where 10 μL of [^{33}P]-labeled 12 base pair DNA was incubated with 50 μL 0.1 M NaCl in 50 mM HEPES, pH 7.4, and after 10 to 15 minutes the stop solution (50 μL) was added. After the addition of the stop solution, all samples were processed similarly according to the steps described in the procedure (6).

Sequencing Gel

The apparatus required for sequencing the DNA comprises two glass plates (20 cm x 19 cm and 20 cm x 15 cm) and side and bottom spacers. The spacers' thickness determines the thickness of the gel and 0.5 mm spacers were used. The spacers were greased with silicon oil and placed between the glass plates, which were then clamped to tighten the frame.

The separation of DNA fragments is performed on acrylamide-urea gels (6). For cleaved DNA products smaller than 30 nucleotides, the gel comprises 19% (w/v) acrylamide, 1% (w/v) bisacryamide, 8.3 M urea, 100 mM tris-borate, pH 8.3, and 2 mM EDTA.

A mixture of these materials was mildly heated and stirred until the urea was

completely dissolved. To this solution, 200 μL of 10% (w/v) ammonium persulfate was added, and the mixture was degassed for 5 minutes. In order to plug the bottom of the gel, 1 mL of the solution was removed to a test tube and 2 μL of TEMED was added to it. The solution from the tube was then poured in between the glass plates to form the plug. Three minutes later, after the polymerization stage, the rest of the solution was added, after being mixed with 15 μL of TEMED. A comb was placed at the top to create the wells within which the DNA would be loaded.

It took approximately 10 to 15 minutes for the polymerization to conclude and for the gel to be ready (the indication is usually the polymerization of the solution remaining in the flask). The plates were placed in the electrophoresis apparatus and the buffer cases were filled with 1 liter of 100 mM tris-borate buffer pH 8.3 and 2 mM EDTA solution (TBE).

Pre-electrophoresis of the gel, to eliminate salt and ion contaminations, was conducted for 1 hour at 300 volts. After loading the gel with the DNA, the samples were electrophoresed at 400 volts for 2 hours and 30 minutes.

The autoradiography method was used to determine the location of the radioactive material on the gel. The gel was removed from the glass plates, and wrapped with a saran wrap. The gel was then placed in an a light impenetrable folder. A Fuji GBX film was placed on top of the gel to record the emission of the radioactive material.

Film Development

For the development process, the GBX developer and fixer solutions were diluted at a 1:5 dilution ratio. During the process the safety light was turned on and the solutions were placed in three trays; one containing the developer, another, 3% acetic acid, and a third, the fixer. The film was placed in the developer for 5 minutes, transferred to the acetic acid solution for 1 minute (to stop the development process), and then to the fixer for an additional 5 minutes. At the end of this procedure, lights may be turned on. The film was placed under running water for 15 minutes and then hung up to dry.

An experiment was designed to examine the films' quality, the safety light in the dark room and its effect on the films, the development process and the folders in which the gels and the films were placed. The three solutions were placed in separate trays. The safety light was turned on and one film was exposed to the safety light for 1 minute. The film was then placed in the developer, transferred to the acetic acid solution, and then to the fixer as previously described. At the end of this procedure, the light was turned on and the film was placed under running water for 15 minutes.

The same protocol was repeated with no safety light throughout the procedure to evaluate the effect of the safety light on the film. The results obtained showed no difference between the two films, indicating that the safety light could be left on during the development process. In order to verify that light cannot penetrate the folders, the folders were tested prior to experimental use.

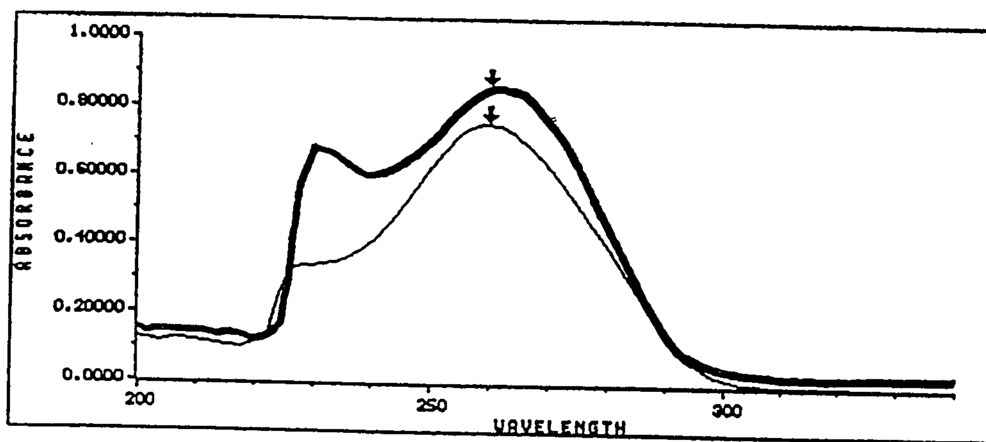
Results

Protocol Verification

The Stability of Double Stranded DNA

This experiment was designed to determine the minimal length of DNA oligomer, which would still be stable in a double stranded form at room temperature and neutral pH (50 mM HEPES, pH 7.4). The experiment is based on the principle that the absorbency of single stranded DNA is higher than that of double stranded DNA helices (16). Therefore, by comparing the absorbency of a DNA sample before and after denaturation, it is possible to conclude, whether or not the DNA was in the double stranded form before the denaturation process. Since at known concentration ratios, the ratio between the molar absorptivities or extinction coefficients (denatured and native) is proportional to the ratio between the absorbencies, it can be used to indicate stability.

DNA strands of 10, 12, 14, and 22 oligonucleotides in size were prepared to produce approximately 50 μg/mL DNA in 50 mM HEPES and were characterized using UV absorbency. The UV spectra of 12 base pair double stranded DNA in 50 mM HEPES and single stranded DNA of 12 nucleotides in size after addition of 3 M NaOH to 0.2 M are presented in **Figure 8**. The measured absorbance at 260 nm for the double stranded DNA (native) ranged between 0.25 to 1.00 and for the denatured DNA between 0.26 to 1.10. The experiments were conducted several times and the average results with their standard deviations are reported in **Table 2**. **Figure 9** is a graphic presentation of the



Marked Wavelengths
Reg A: L 260 = 0.75438

Marked Wavelengths
Reg A: L 260 = 0.86263

Figure 8: UV spectra of 12 base pair oligomer DNA in 50 mM HEPES (—) and in sample after addition of 3 M NaOH to 0.2 M (—).

DNA Size	A_{260} in 50 mM HEPES	A_{260} after addition of 3 M NaOH solution to 0.2 M.	Extinction Coefficient Ratio, Avg.[$\varepsilon_{alkali}/\varepsilon_{neutral}$] ± std. dev.
10	0.65414	0.55203	0.999 ±0.082
12	0.75438	0.86263	1.179 ±0.051
14	0.26279	0.26105	1.124 ±0.072
22	0.89894	1.0294	1.178 ±0.060
ss 12	1.2116	1.1736	1.033

Table 2: The average and standard deviation values of the extinction coefficient ratios, $\varepsilon_{alkali}/\varepsilon_{neutral}$, calculated from four trials, as a function of the DNA size.

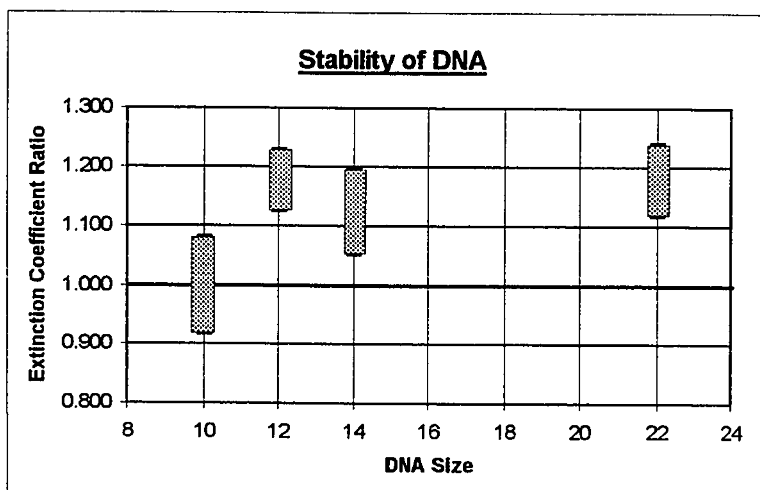


Figure 9: Extinction coefficient ratios, $\varepsilon_{alkali}/\varepsilon_{neutral}$, as a function of DNA size. The shaded regions represent the standard deviations of $\varepsilon_{alkali}/\varepsilon_{neutral}$.

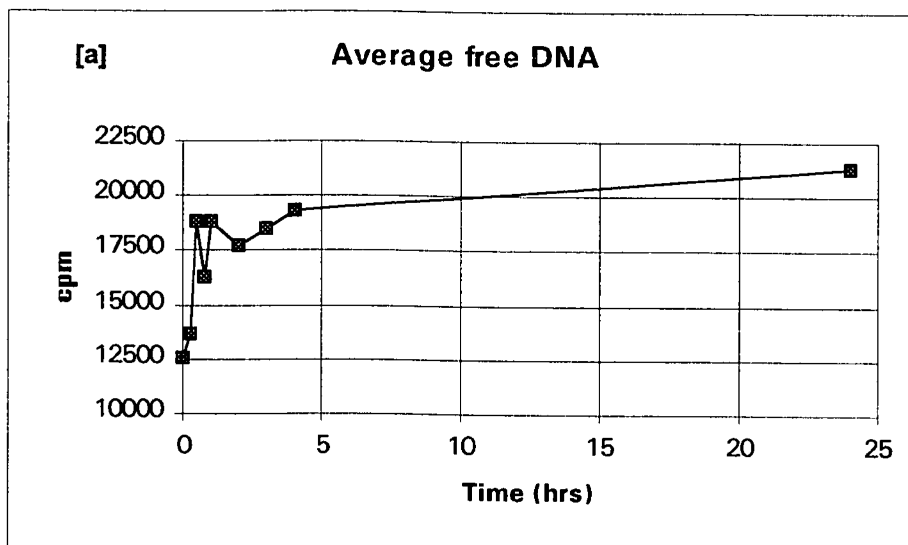
results in Table 2. The control for the stability experiment was conducted with single stranded DNA which was denatured with 0.2 M NaOH solution. Very little change was obtained in the ratio of extinction coefficient ($\varepsilon_{alkali}/\varepsilon_{neutral} \cong 1$) as was expected. This shows that little change in absorptivity for single stranded DNA is observed as the pH changes from neutral to alkaline conditions.

The results revealed that the range of values for the extinction coefficients' ratio for the DNA of 12 to 22 oligomers in size were always greater than one (the averages' ratios were from 12 to 17% higher), indicating their stability in the double stranded form. The range of values, however, for the 10 base pair oligomer fluctuates above and below one, indicating on average its presence as single stranded DNA under the conditions chosen.

The Dissociation of the DNA-Protamine Complexes

The objectives of these experiments were to verify the reliability of the DNA-protamine dissociation protocol (33) and to adapt this protocol to the methylation protection experiment. For the experiments' controls it was necessary to measure the non-specific binding of the DNA to the filter paper and to the unsubstituted resin.

Dissociation of the complexes was examined at various time intervals after incubation of twelve base pair oligomer [^{33}P]-end-labeled DNA, was incubated with 200 µL of a low substituted resin (12.6 pmol protamine/10 mg resin). As presented in **Figure 10**, the results showed an increase in the amount of free DNA and a decrease in the amount of bound DNA with time. The results of controls with either free DNA or DNA



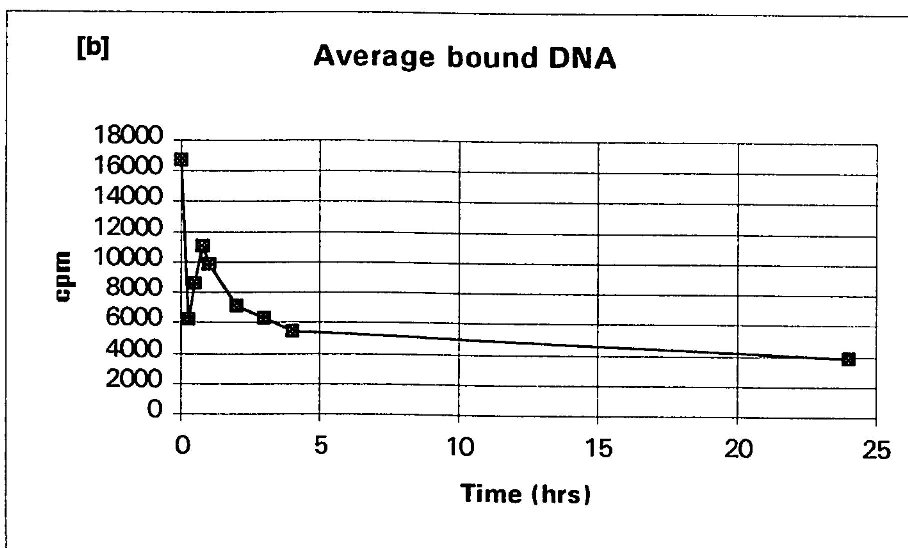


Figure 10: **The dissociation of 12 base pair oligomer (specific activity of 0.46 μCi/pmol DNA) from P1 substituted resin in 0.1 M NaCl in 50 mM HEPES pH 7.4. [a] The average cpm of the free DNA as a function of time (hours); [b] The average cpm of the bound DNA as a function of time (hours). Fifty six percent (56%) of the DNA is initially bound, and 16% remained bound after 24 hours. Calculated dissociation rate constant $k_2 = 1.1 \times 10^{-5}$.**

added to unsubstituted resin showed very low non-specific binding in which approximately 2 to 4% of the labeled DNA remained bound either to the filter or to the resin. The data were consistent with previous results (33), and sufficient reversible binding occurred to allow the use of these materials to perform a methylation protection experiment. The results showed that the DNA-protamine complexes were formed during the incubation period, and the binding between the DNA and the protamine was reversible (i.e., dissociation was observed with time).

The formation of the complex as well as the dissociation process are both crucial for the methylation protection experiment. It was necessary to adapt the original methylation experiment protocol to include dissociation. The purpose of the following experiments is to develop a methylation protection experiment protocol, which would best suit the DNA-protamine system. In designing these experiments, one has to consider the behavior of the complexes and the optimal environment for the methylation to occur.

In the methylation protection experiment, the first step after the formation of the complexes is the methylation by the DMS (dimethyl sulfate) reagent, followed by removal of DMS and then the dissociation of the bound DNA from the P1 protamine. Since only limited methylation of the DNA is required, a stop solution has to be added to the mixture in order to stop the process, suggesting that the stop solution and the dissociation solution could be one and the same.

Previous dissociation experiments (33) showed that 2 M sodium chloride (NaCl) provides rapid and efficient dissociation. Since the methylation protocol (6) contains a stop solution with 3 M sodium acetate (NaOAc), the ability of this salt to be a good

dissociation solution was tested. The choice was made since the concentration of 3 M gives a high ionic strength and the 3 M NaOAc is normally used with 95% ethanol to precipitate DNA (6).

The following experiments were conducted with 200 µL low substituted resin (12.6 pmol/ 10 mg resin) incubated with [^{33}P]-labeled DNA and dissociated in a 0.5 mL of 3 M NaOAc solution at various time intervals (0, 15, 30, 45, and 60 minutes). The results were compared with the dissociation in 0.5 mL of 2 M NaCl in 50 mM HEPES buffer as shown in **Figure 11.**

Measurements of the amount of DNA bound at 0 time by dissociation in 0.1 M NaCl in 50 mM HEPES pH 7.4 were not taken here; however, a comparison with the dissociation experiment conducted in 0.1 M NaCl solution, where 56% of the DNA was initially bound (Figure 10), showed that there was no evidence of dissociation in the 3 M NaOAc solution with an approximately constant percentage of 35% of bound DNA was measured at all times. There is a possibility that about 15% of the bound DNA was immediately released (compared with 56% bound at 0 time, Figure 10) and no more dissociation was obtained during longer dissociation times. In contrast, however, immediate and efficient dissociation, compared to the results presented in Figure 10, was observed for the 2 M NaCl solution with about 10% of the DNA remaining bound after dissociation.

It was noted that the 3 M NaOAc and the 2 M NaCl in 50 mM HEPES dissociation solutions showed a difference in pH. The pH of the 3 M NaOAc was 8.3 and the pH of the 2 M NaCl solution was 7.4 (the buffer pH), which may have caused the

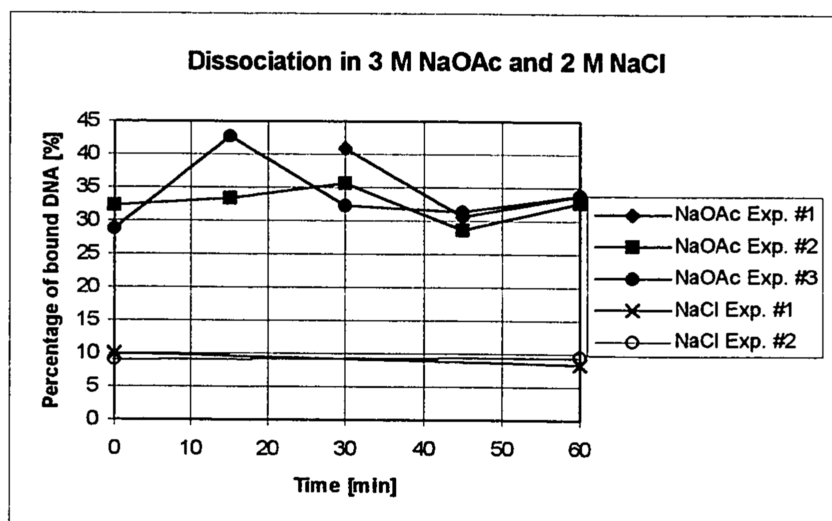


Figure 11: The percentage of bound DNA as function of time in 0.5 mL of 3 M NaOAc (◆, ■, ●) and 0.5 mL of 2 M NaCl (x, o).

difference in the observed dissociation of the complexes. Dissociation experiments in 3 M NaOAc in a 50 mM HEPES buffer, pH 7.4 (pH adjusted to 7.4 with 50 mM HEPES acid), and in 2 M NaCl in a 50 mM HEPES buffer, pH 8.3, were conducted for the determination of the pH effect on the dissociation process. The results, shown in **Figure 12**, were very similar to the previous ones, suggesting that these pH differences are not responsible for the different extents of dissociation. From these experiments, it was concluded that the 3 M NaOAc solution is not effective as a dissociation solution, and can not be used directly in the methylation protection experiment.

Since the 3 M NaOAc solution had not caused sufficient dissociation, it was decided to use a solution with a divalent cation, 3 M $Mg(OAc)_2$, which may better displace the DNA from the protamine, and would thus provide a more effective dissociation solution.

The dissociation experiments were conducted in 0.5 mL of 3 M $Mg(OAc)_2$. The results, shown in **Figure 13,** exhibited yet again a constant percentage of bound DNA and no obvious dissociation. In addition, compared to the NaOAc solution, a higher percentage of DNA seemed to be bound to the protamine. Furthermore, the viscosity of the solution was observed to be very large and the filtration time visibly took much longer.

At this stage, in an attempt to explain the previous results, the effect of the various solutions on the non-specific binding of the DNA to the filter paper and to the resin was measured. The non-specific binding to the filter paper was examined by the direct filtration of the labeled DNA mixed in the various solutions. The non-specific binding to the resin was tested by the incubation of the unsubstituted resin in the various solutions, followed

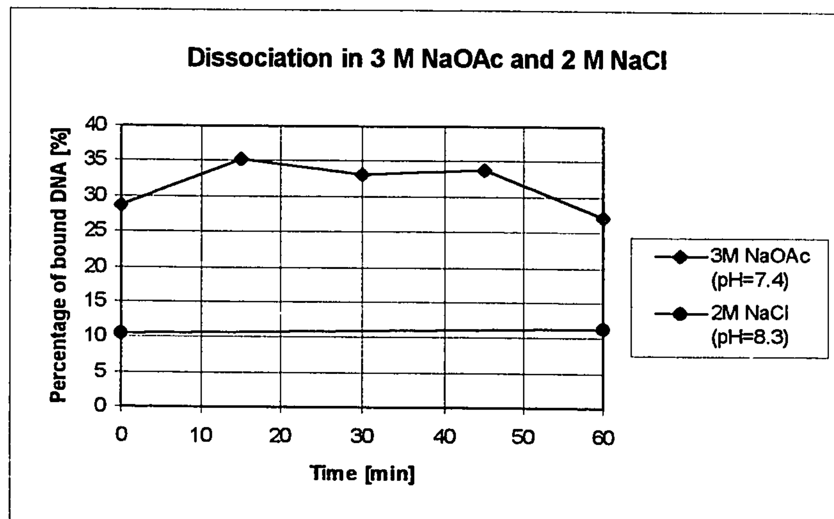


Figure 12: Dissociation in 0.5 mL of 3 M NaOAc in 50 mM HEPES pH 7.4 (◆) and in 0.5 mL 2 M NaCl in 50 mM HEPES pH 8.3 (●).

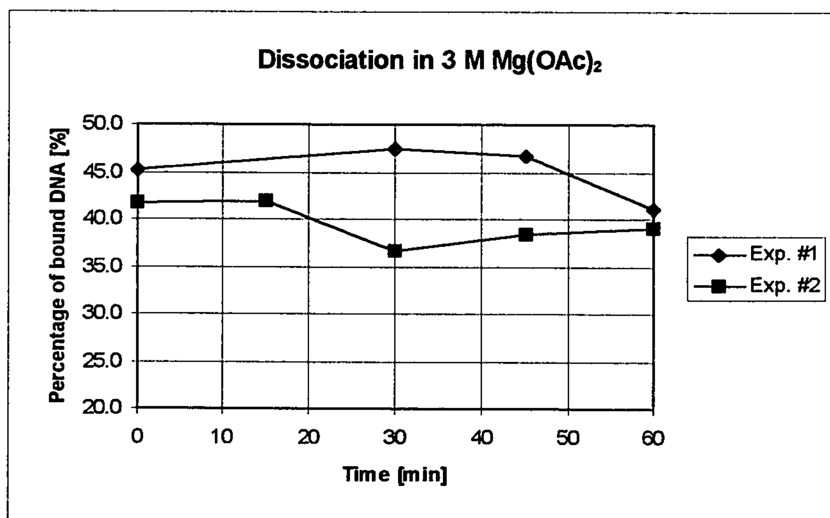


Figure 13: Dissociation of 12 base pair DNA in 3 M $Mg(OAc)_2$. The percentage of bound DNA as function of time (min).

by filtration. Two samples of each solution were examined at 0 and 30 minutes. The results were compared with the dissociation from the low substituted resin in the same solutions at similar time intervals. The results are shown in **Table 3** and **Figure 14**.

The results exhibited, that the non-specific binding of the DNA directly to the filter paper had an average of 22% in the 3 M $Mg(OAc)_2$ solution, 10% in the 3 M NaOAc solution, 2.5% in the 2 M NaCl solution and 1.8% in the 0.1 M NaCl solution. The non-specific binding of the DNA to the unsubstituted resin and the filter paper had an average of 32% in the 3 M $Mg(OAc)_2$ solution, 26% in the 3 M NaOAc solution, 4% in the 2 M NaCl solution and 4% in the 0.1 M NaCl solution. It became clear at this stage that neither 3 M $Mg(OAc)_2$ nor 3 M NaOAc could be used as an appropriate dissociation solution.

Since the NaCl solution would be used in the methylation protection experiment to dissociate the DNA-protamine complexes, the only remaining issue was to determine the appropriate NaCl concentration for the immediate dissociation step in this experiment. The next experiment was designed to test the effectiveness of a 0.1 M NaCl solution as a dissociation solution with resin volumes of 50 μL (called for in the protocol) and 10 μL (used in the dissociation experiments). The results presented in **Figure 15** show dissociation with 0.1 M NaCl in HEPES pH 7.4 of 10 μL and 50 μL mixtures of DNA-protamine complexes.

The results revealed that 0.1 M NaCl solution is not very effective in the immediate dissociation from low substituted resin in large volumes (Figure 15) or in small volumes (Figures 10 and 15). The 2 M NaCl solution was found to be a good dissociation

3 M $Mg(OAc)_2$	time (min)	free DNA	bound DNA	total cpm	% bound
Direct	0		185403	824196	22.5
	30		176792	824196	21.5
Unsubstituted	0	18096.6	89488.2	270454	33.1
Resin	30	23133.4	105182	336516	31.3
Substituted	0	27034	131660	402000	32.8
Resin	30	21410	113124	327224	34.6
3 M NaOAc	**time (min)**	**free DNA**	**bound DNA**	**total cpm**	**% bound**
Direct	0		43063.2	386133	11.2
	30		36705.2	386133	9.5
Unsubstituted	0	25041	87876.4	338286	26.0
Resin	30	21688	77174.6	294054	26.2
Substituted	0	25205.6	95733	347789	27.5
Resin	30	17491.6	65053.2	239969	27.1
2 M NaCl	**time (min)**	**free DNA**	**bound DNA**	**total cpm**	**% bound**
Direct	0		21896.8	824196	2.7
	30		20038.4	824196	2.4
Unsubstituted	0	18835	7303.4	195653	3.7
Resin	30	23520.6	10822.8	246028	4.4
Substituted	0	20441.8	15795.6	220213	7.2
Resin	30	17852.6	12487.6	191013	6.5
0.1 M NaCl	**time (min)**	**free DNA**	**bound DNA**	**total cpm**	**% bound**
Direct	0		6507.4	386133	1.7
	30		7414.6	386133	1.9
Unsubstituted	0	24407.6	9100	253176	3.6
Resin	30	14647.4	7285.6	153759	4.7
Substituted	0	13623.6	23630	159866	14.8
Resin	30	20097.2	32644	233616	14.0

Table 3: The non-specific binding to the filter paper and the unsubstituted resin in comparison with the dissociation from the substituted resin. Dissociation in 3 M $Mg(OAc)_2$, 3M NaOAc, 2 M NaCl, and 0.1 M NaCl.

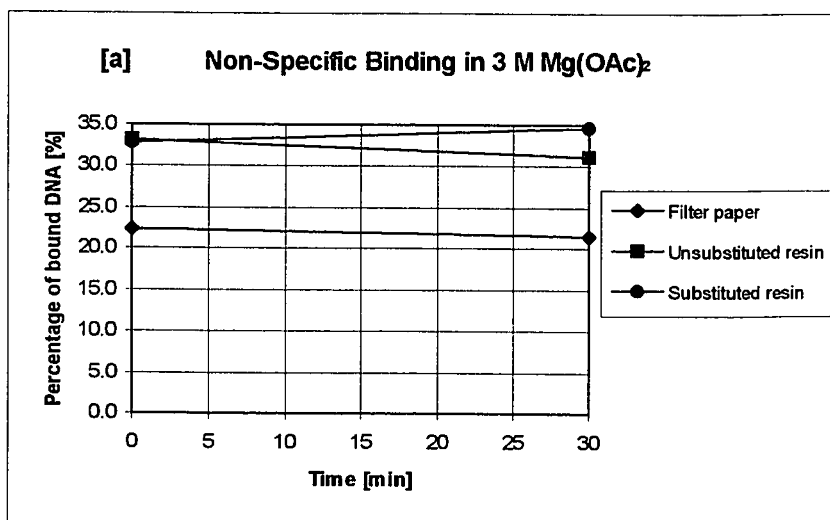


Figure 14a

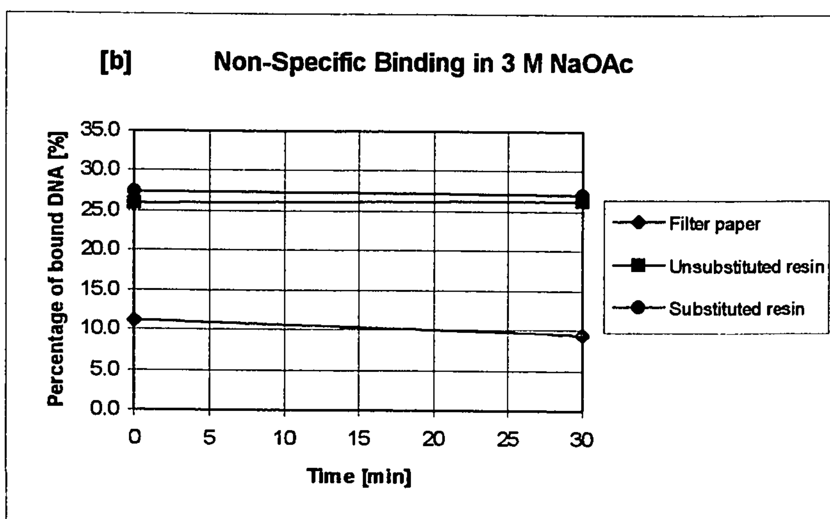


Figure 14b

Figure 14: The non-specific binding of the DNA to the filter paper (◆), binding to the unsubstituted resin (■), and binding to the substituted resin (●) in: [a] 0.5 mL of 3 M $Mg(OAc)_2$; [b] 0.5 mL of 3 M NaOAc; [c] 0.5 mL of 2 M NaCl; [d] 0.5 mL of 0.1 M NaCl.

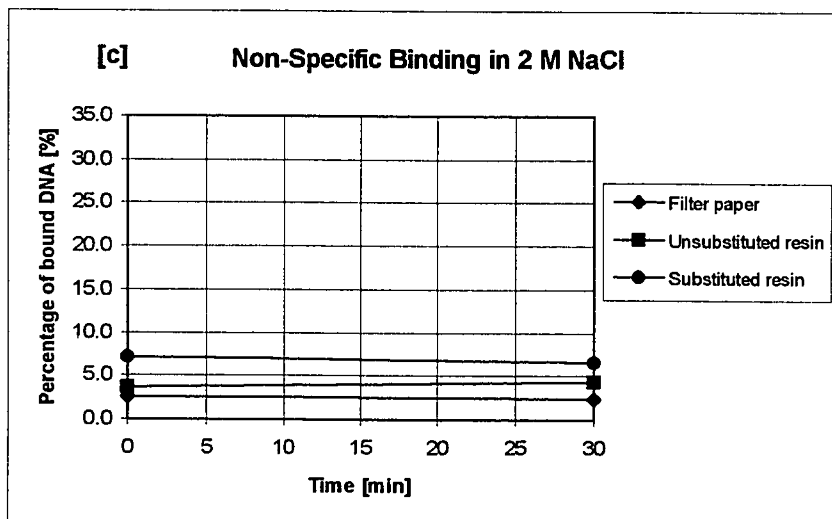


Figure 14c

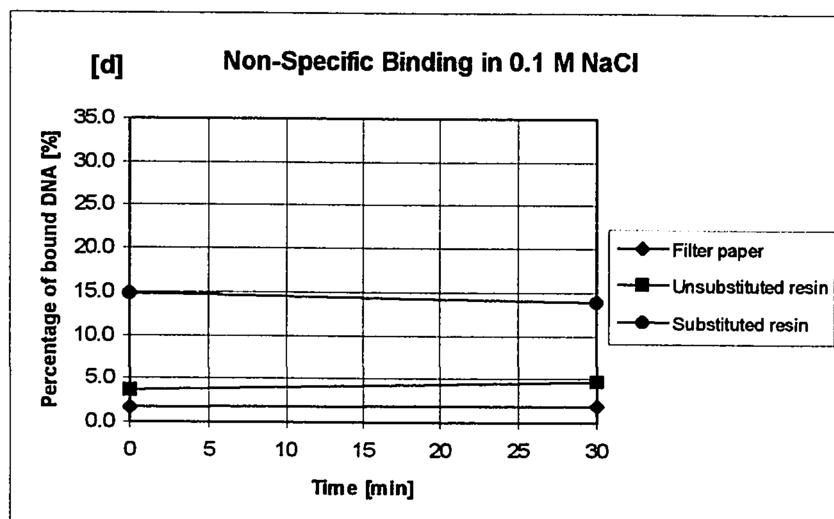


Figure 14d

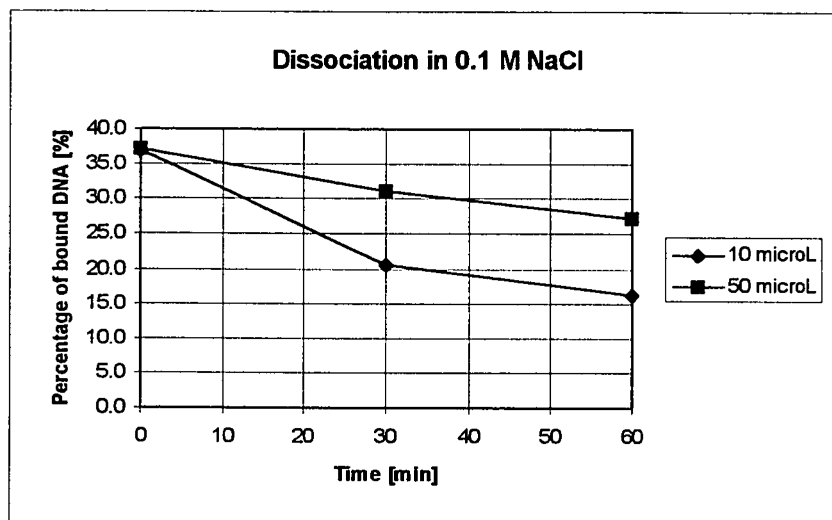


Figure 15: Dissociation of 10 μL (◆) and 50 μL (■) complexes in 0.1 M NaCl in 50 mM HEPES pH 7.4.

solution of complexes in large volumes (50 μL) (Figure 11 and 12), since efficient and rapid dissociation was obtained immediately after applying the complexes to the solution.

The Gel

The purpose of this experiment was to check the loading technique and to determine the film exposure time sufficient for registering the DNA counts. For this, two gels were prepared, and various amounts of [^{33}P]-labeled 12 base pair DNA were loaded - with approximately 8 x 10^4 cpm, 5 x 10^5 cpm, 1 x 10^6 cpm, and 2 x 10^6 cpm in separate lanes. After electrophoresis, each gel was placed in a folder, with an overlaying X-ray film. The exposure period was set to 16 hours for one of the folders and 24 hours for the other.

The results revealed three lanes each containing one band with the same migration distance and an increase in the intensity of the band according to the amount of radioactive material added (data not shown). However, in the lanes containing the 8 x 10^4 cpm, no bands were apparent. These gels were placed in the folders with new films, this time for exposure of 6 days. The film was developed and a band appeared in the gels for the lanes containing 8 x 10^4 cpm, both exhibiting similar migration distances (data not shown). These results indicated that for approximately 5 x 10^5 cpm or more per one band, 16 to 24 hours of exposure are sufficient for the bands to register on the autoradiogram. When one band contains 8 x 10^4 cpm or less, the film has to be exposed to the radioactive material for longer time periods. The similar migration distance of the oligonucleotides with the same size indicated that the gel conditions are appropriate for the DNA sequencing.

Methylation and Cleavage

The objectives of this group of experiments were to verify the methylation and cleavage protocols proposed by Maxam and Gilbert (6) and to develop a protocol appropriate for the DNA-protamine system. To verify the success of methylation and cleavage, several bands migrating faster than the 12 base pair oligomer band should appear on the autoradiogram. These bands would indicate smaller fragments of DNA obtained after cleavage.

Results of preliminary experiments according to the original Maxam and Gilbert (6) protocol, showed only a 5 to 7% loss of cpm during the washes, and yet at the same time, no bands were apparent on the autoradiogram. There was a possibility that the amount of radioactive material actually loaded on the gel at the end of the procedure was too small to register on the film. It was important, therefore, to determine the amount of labeled DNA in the sample to be loaded. This was accomplished by counting 1 μL of each processed sample before loading on the gel.

Two samples of 80 ng of free 10 base pair DNA (1.1×10^7 cpm) were processed completely, methylated and cleaved. During the washes, 47 to 58% of the material was lost, which was much more than observed in the preliminary experiments. However, more radioactive material in terms of actual counts was loaded on the gel at the end of the process. After the cleavage reaction the samples were loaded on the gel and the exact cpm loaded was determined. The samples' tubes were washed after the loading in order to verify the amount of radioactive material left in them, and only 0.9% to 1.1% of the cpm

were lost in this step.

After 3 days of exposure, the following results were observed as shown in the autoradiogram presented in **Figure 16**. In lanes 1 and 2, a few bands appeared, corresponding to the migration distance of 10 base pair DNA and probable cleavage products, which migrated faster. Lanes 3 and 4 were controls, therefore, three bands appeared in each of them; one band, which correlates with the 12 base pair, one with the 10 base pair and one with the 7 base pair DNA.

These results confirmed that the complete procedure works, and that the intensity of the bands is directly related to the initial amount of labeled DNA used. In order to obtain darker bands, larger amounts of labeled DNA have to be present in each cleavage band. Therefore, the methylation and cleavage protocol from this point onwards was conducted with 10 μL (80 ng, 10^7 cpm) of the labeled DNA.

Although bands appeared on the film, a large amount of radioactive material remained at the origin. To overcome this problem, the samples were heated in a water bath at 90 °C for one minute before loading on the gel. The experiments demonstrated that the modified protocol was appropriate for the investigation of protection from methylation of DNA grooves by protamine.

The determination of the size of DNA fragments obtained from the cleavage process was performed by a comparison to a set of standards' migration on the sequencing gel. A mixture of nucleotides of even numbers, 6 to 20 nucleotides in size, was labeled, and electrophoresed on the gel as a marker for the results. **Figure 17** shows the migration of the standards. The bands were clearly resolved. In addition, a mixture of odd numbers

Figure 16: Autoradiogram of methylated and cleaved 12 base pair DNA. The lanes are numbered from left to right. Lanes 1 and 2 are two individual samples of methylated and cleaved 12 base pair DNA. Lane 1 contains 1×10^5 cpm and lane 2 contains 1.7×10^5 cpm and 10 base pair DNA (1.1×10^6 cpm). Lanes 3 and 4 are controls. Lane 3 contains 7 base pair DNA (7.2×10^5 cpm), 10 base pair DNA (1.1×10^6 cpm), and 12 base pair DNA (1.1×10^5 cpm). Lane 4 contains 7 base pair DNA (7.2×10^5 cpm), 10 base pair DNA (1.1×10^6 cpm), and 12 base pair DNA (1.1×10^5 cpm).

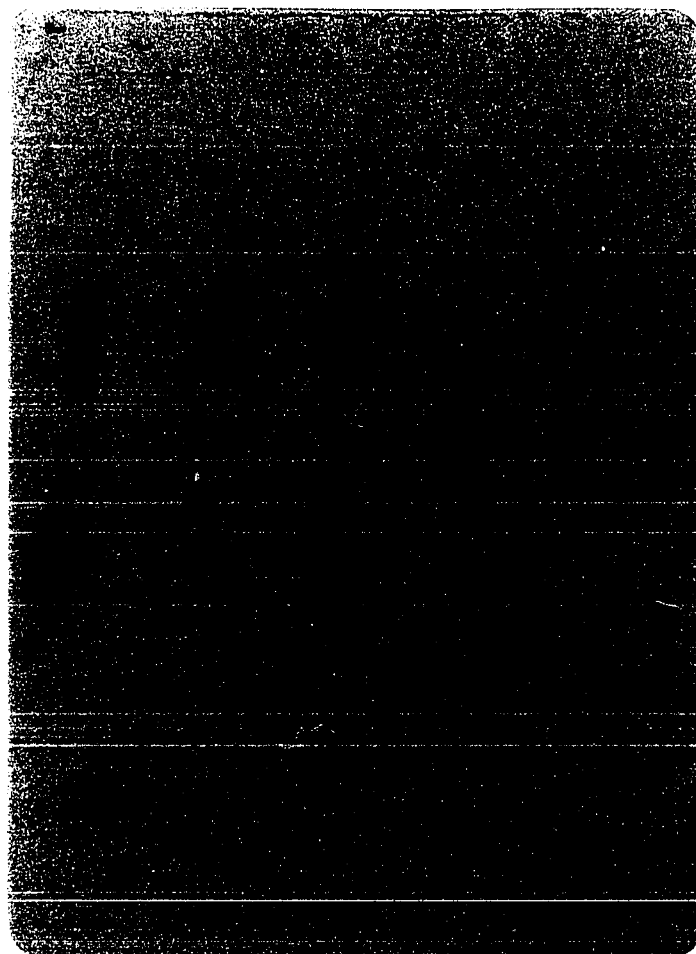


Figure 17: Autoradiogram of various amounts of labeled even standards of 6 to 20 nucleotides in size. The lanes are numbered from left to right. 1.0 μL (3.6×10^5 cpm), 0.5 μL (1.8×10^5 cpm), and 1.5 μL (5.5×10^5 cpm) in lanes 1, 2, and 3, respectively. A sample of 12 base pair DNA is shown in lane 4. The film was exposed for 48 hours.

of nucleotides, 5 to 19 in size, was labeled and electrophoresed as well. Together, these markers provide a standard curve of the migration distance to be utilized in the analysis of the cleaved products.

The migration distances of DNA fragments of the same sizes are assumed to be very similar if not identical. The migration is a function of the length, the net charge and the shape of the molecule of interest. The larger the oligonucleotide chain, the smaller the migration distance. **Table 4** lists the migration distances measured for standards of 5 to 20 oligonucleotides in size and **Figure 18** represents the plot of migration distance vs. the logarithm of the DNA size. There is an inverse correlation between the logarithm of the molecular weight and the migration distance or between the logarithm of the DNA size and the migration distance. **Table 5** presents the calculated migration distances for 12 to 1 oligomers in size, based on the linear regression curve from Figure 18.

Localization of Protamine Binding on the DNA

The following experiments were conducted with the purpose of obtaining sufficient data, which would explicitly point to the binding site of the protamine to the DNA. All the previous experiments helped design and verify the steps, which led to the modified protocol for these experiments.

As described in the modified protocol, DNA incubated with protamine-substituted resin was used for the methylation protection experiments. After methylation with DMS and removal of the free DNA, dissociation of the bound DNA was obtained with the stop solution containing 4 M NaCl, and both DNA samples were processed for piperidine

DNA size	log[DNA size]	Migration Distance [mm]
20	1.301	42
19	1.279	44
18	1.255	45
17	1.230	47
16	1.204	48
15	1.176	51
14	1.146	51.5
13	1.114	54.5
12	1.079	55.5
11	1.041	59.5
10	1.000	60
9	0.954	65
8	0.903	66
7	0.845	70
6	0.778	72
5	0.699	78

Table 4: The DNA size and the migration distance measured from the autoradiogram presented in Figure 17.

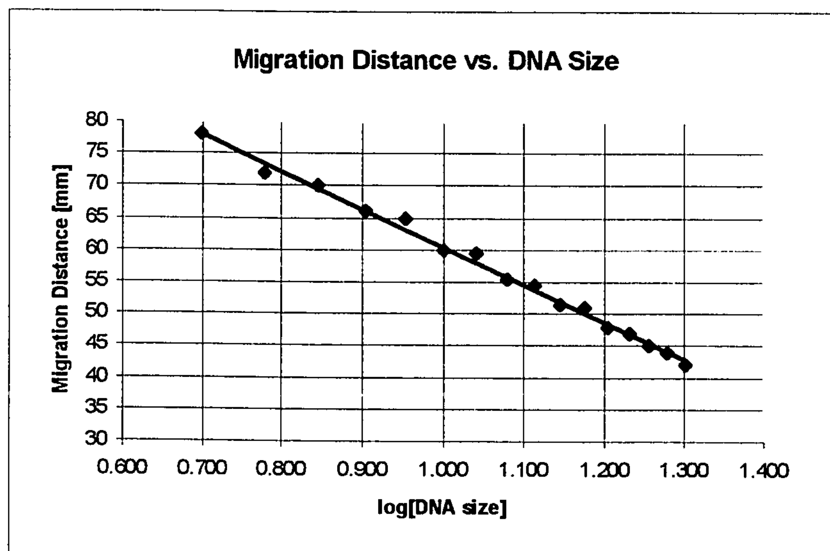


Figure 18: The migration distance in mm as a function of the logarithm of the DNA size (linear regression line is shown).

DNA size	log[DNA size]	calculated migration distance [mm]
12	1.079	55.9
11	1.041	58.1
10	1.000	60.5
9	0.954	63.2
8	0.903	66.2
7	0.845	69.6
6	0.778	73.6
5	0.699	78.2
4	0.602	83.9
3	0.477	91.3
2	0.301	101.6
1	0.000	119.3

Table 5: Calculated migration distances for DNA fragments of 12 to 1 oligomers in size, based on the extrapolated linear regression from Figure 18.

cleavage. The cleaved DNA products were loaded on the sequencing gel, such that the standards to be used as references were loaded in the adjacent lanes on both sides of each sample.

The results were analyzed by comparing the cleavage products of the methylated bound 12 base pair DNA with those of the methylated free 12 base pair DNA, which were treated in the same manner, subsequent to their separation after methylation. The resulting cleavage pattern of both the bound and the free DNA samples are expected to be different, since more sites of the free DNA are exposed for methylation. In this case based on the sequence of the 12 base pair DNA, a cleavage pattern of dark bands (methylation of guanine) of 2, 6 and 10 oligomers in size and lighter bands (methylation of adenine) of 3, 4, 8, and 11 oligomers in size are expected to be found (4 and 11 oligomers in size are products from both strands of DNA, which may increase the relative intensity of these bands). For the bound DNA, the expected results depend on the binding site. Binding to the major groove should produce bands of 3, 4, 6, 8, 10, and 11 oligomers in size, and binding to the minor groove should produce bands of 2, 6 and 10 oligomers.

Figure 19 presents the results of the methylation experiments for the free and the protamine-bound DNA. The results obtained from the methylation protection experiment revealed that three samples of bound DNA, each prepared separately (lanes 2, 4, and 6), appeared to have identical cleavage patterns. The free DNA (lane 8) had considerably more cpm and thus, cannot be directly compared with the bound DNA in terms of the intensities. However, the cleavage pattern of the free and the bound DNA did appear to be

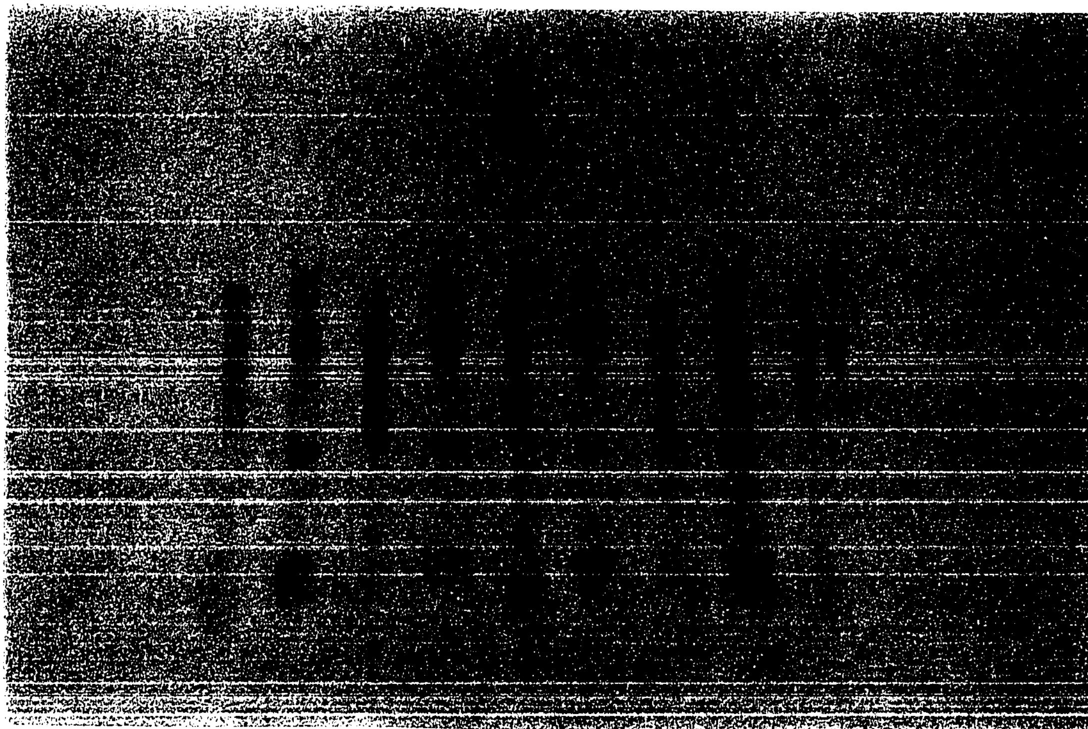


Figure 19: Autoradiogram of free and bound DNA. The lanes are numbered from left to right. Lanes 1, 5, 9 contain even standards (6 to 20 oligomers in size). Lanes 3, 7 contain odd standards (5 to 19 oligomers in size). Lanes 2, 4, 6 contain bound DNA cleaved products (7.9×10^4 cpm, 7.6×10^4 cpm, 7.3×10^4 cpm, respectively). Lane 8 contains free DNA cleaved products (3.4×10^5 cpm), and lane 10, a 12 base pair DNA (7.9×10^4 cpm). The film was exposed for 3 days.

very similar. In both cases, the darker bands appeared at migration distances of 58 mm, 65 mm, and 81 mm, which according to Table 5 corresponds to fragments of 12, 8, and 4 oligonucleotides, respectively, and another weak band at 62 mm is observed, which corresponds to a 10 oligonucleotide chain.

Figure 20 shows the results of another methylation and cleavage experiment with labeled 12 base pair DNA. Again, the samples were prepared separately and the cleavage pattern of the bound DNA (lanes 2 and 6) was very similar to that of the free DNA (lanes 4 and 8). The intensity of the bands cannot be compared since the cpm for the free DNA is much higher than the bound DNA. The migration distances corresponded to fragments of 12, 10, 8, and 4 nucleotides in size, according to Table 5. These results were fully in agreement with those of the previous experiment (Figure 19).

Another methylation and cleavage experiment was performed, this time using free DNA of various known sequences and sizes (7, 10 and 12 oligonucleotides), to see whether the results would yield the expected cleavage pattern, that is, intense bands for cleavage next to guanines and less intense bands resulting from cleavage next to adenines. The protocol used was similar to the previous ones, with the exception that protamine was not involved. **Table 6** presents the measured migration distances of the cleavage products, and **Figure 21** shows the autoradiogram presenting these results. Comparing the migration distances to the standards (Table 5), the cleavage pattern of the 7 base pair oligomer contains more bands than expected (including bands which migrated slower than the uncleaved 7 base pair sample). However, the faster migrating bands corresponding to

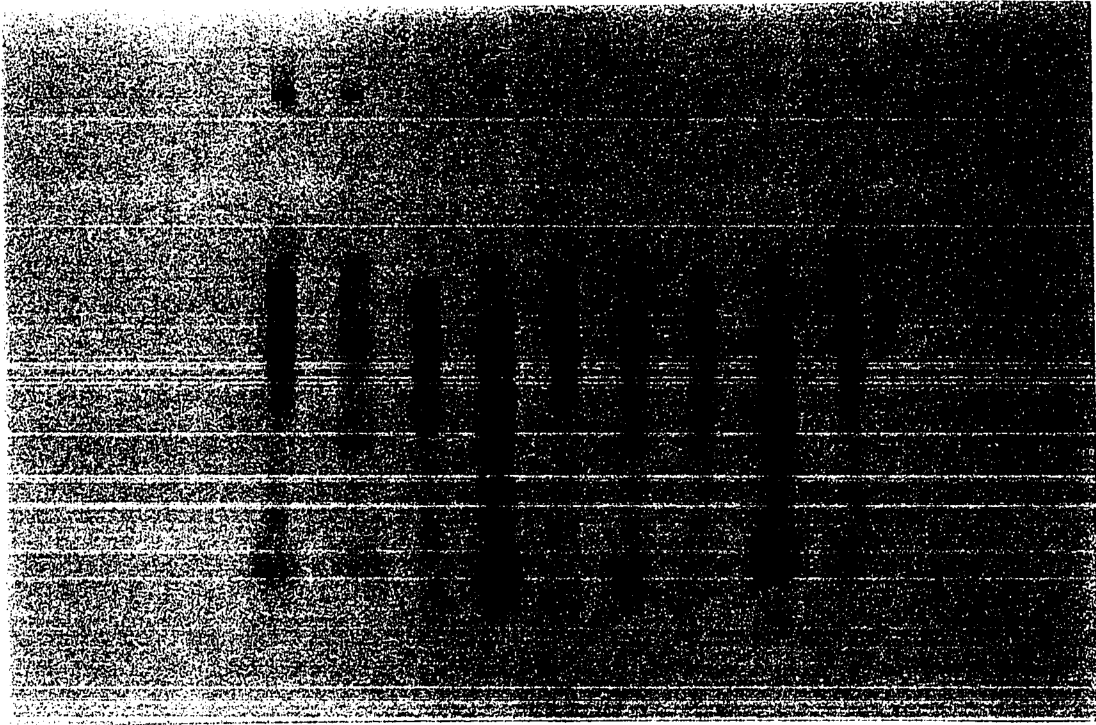


Figure 20: Autoradiogram of bound and free DNA. The lanes are numbered from left to right. Lanes 1, 5, 9 contain even standards (6 to 20 oligomers in size). Lanes 3, 7 contain odd standards (5 to 19 oligomers in size). Lanes 2, 6 contain bound DNA products (4×10^4 cpm and 8.6×10^4 cpm, respectively). Lane 4, 8 contain free DNA products (3.1×10^5 cpm and 2.5×10^5 cpm, respectively), and lane 10, a 12 base pair DNA (6.3×10^5 cpm).

DNA Size	Migration Distance [mm]
7	96, 89, 85, 81, 70, 67, 60, 53, 50, 45.
10	100, 91, 85, 65, 59.
12	55, 59, 62, 76.

Table 6: The measured migration distances of the cleavage products of the 7, 10 and 12 base pair oligomers.

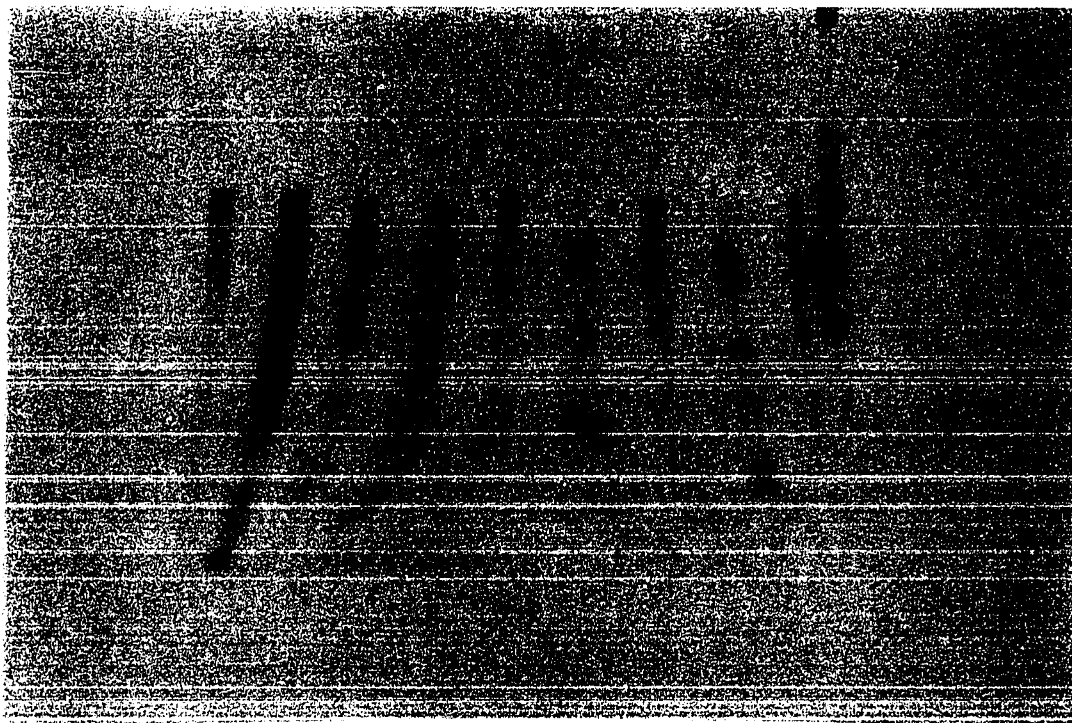


Figure 21: Autoradiogram of various sizes of DNA. The lanes are numbered from left to right. Lanes 1, 5, 9 contain even standards (6 to 20 oligomers in size). Lanes 3, 7 contain odd standards (5 to 19 oligomers in size). In lane 2, cleaved products of 7 base pair oligomer (4.5×10^5 cpm); lane 4, cleaved products of 10 base pair oligomer (5.4×10^5 cpm); lanes 6 and 8, cleaved products of 12 base pair oligomer (3.2×10^4 cpm and 7.1×10^4 cpm, respectively); and in lane 10 a mixture of uncleaved samples 7, 10, and 12 base pair in size (6.4×10^4 cpm, 6.1×10^4 cpm, and 1.5×10^5 cpm, respectively). The autoradiogram was exposed for 6 days.

the expected sizes, such as 7, 5, 4, 3, and 2 nucleotides in size, are present as well. The 10 base pair oligomer shows a partial cleavage pattern (10, 9, 4, 3 and 2 oligomers in size), and the 12 base pair DNA shows a cleavage pattern which corresponds to 12, 10, 8, and 5 oligomers in size.

The bands obtained from the methylation and cleavage reaction (Figures 19, 20, 21) for the free and the bound DNA were not in accordance with the pattern expected from the DNA sequences used (Table 1). The possibility of a shift in the bands' migration was raised. This may be caused by the difference in mass-to-charge ratio between the standards and the cleavage products. The standards used here contain one phosphate group at the 5' end and an OH at the 3' end, whereas the fragments contain phosphate groups on both ends of the chain. Thus, slight alterations in the migration of the cleaved fragments with respect to the standards may occur.

The procedure was conducted again with a 12 base pair oligomer, but this time omitting the methylation step, and thus potentially disabling cleavage. Two samples of 12 base pair DNA were processed according to the modified protocol for free DNA, without the addition of DMS. The migration of the processed 12 base pair DNA was examined for any change in the migration distance due to the procedure. No change in the migration distance was expected. **Table 7** presents the measured migration distances of the cleavage products, and **Figure 22** shows the autoradiogram presenting these results. The methylated and processed 12 base pair DNA (lane 4) showed a cleavage pattern similar to those obtained before, with bands corresponding to 12, 10, 8, 4, and 2 oligomers in size.

DNA Size	Method	Migration Distance [mm]
10	methylated and processed	43, 49, 53, 58, 78.
12	methylated and processed	48, 53, 55, 72, 85.
12	unmethylated and processed	43, 48, 52.
12	unmethylated and unprocessed	48.

Table 7: The measured migration distances of the cleavage products of 10 and 12 base pair oligomers obtained from several methods.

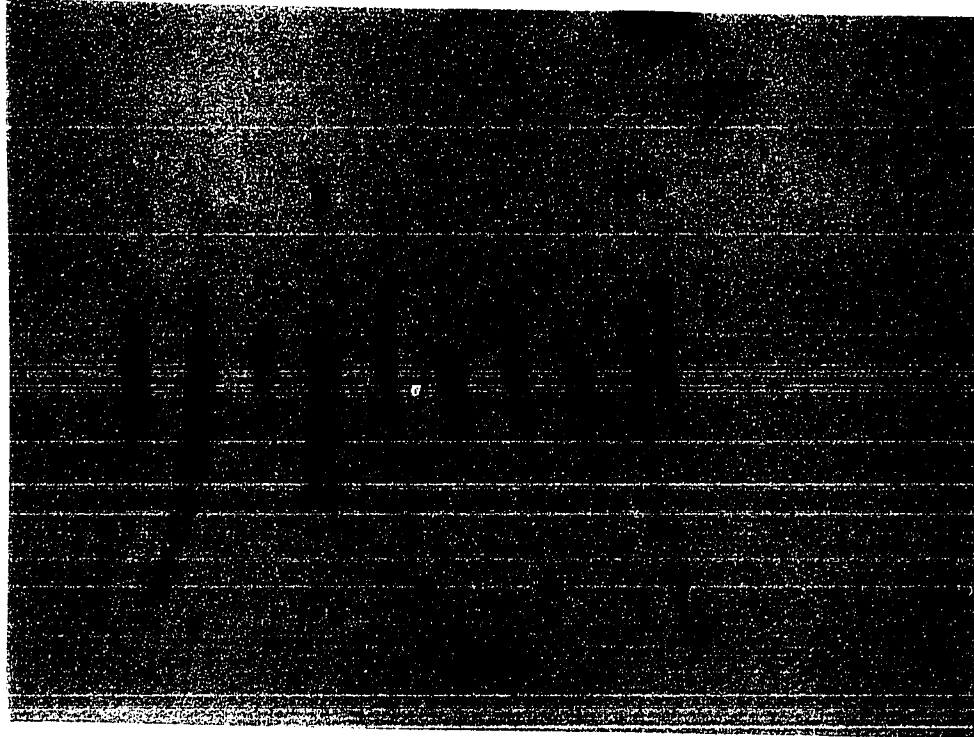


Figure 22: Autoradiogram of [^{33}P]-labeled DNA. The lanes are numbered from left to right. Lanes 1, 5, 9 contain even standards (6 to 20 oligomers in size). Lanes 3, 7 contain odd standards (5 to 19 oligomers in size). In lane 2, methylated and processed 10 base pair oligomer (2.5 x 10^5 cpm); lane 4, methylated and processed 12 base pair DNA (7.3 x 10^5 cpm); lanes 6 and 8, unmethylated and processed 12 base pair DNA (7.4 x 10^4 cpm, and 8.6 x 10^4 cpm, respectively); and lane 10, unmethylated and unprocessed 12 base pair DNA (1.5 x 10^5 cpm). The autoradiogram was exposed for 3 days.

The unmethylated and processed 12 base pair DNA (lanes 6 and 8) showed one band with a migration distance of 12 nucleotides and one band with a migration distance of 10 nucleotides in size. The unmethylated and unprocessed 12 base pair oligomer (lane 10) showed only one band corresponding to the migration distance of a 12-nucleotide chain, as expected.

The similarity between the cleavage pattern of the free DNA and the bound DNA observed in Figures 19 and 20 called for the examination of the stop solution's effectiveness in terminating the methylation reaction. It is possible that the stop solution does not terminate the methylation reaction immediately when added to the complexes, whereas dissociation could begin immediately. The methylation would then continue on the DNA dissociated from the protamine, thus, yielding a cleavage pattern similar to that observed in the free DNA samples.

An experiment was designed to verify the effectiveness of the stop solution in terminating the methylation reaction. One sample consists of a DNA sample added at the same time with the stop solution. The 0.1 M NaCl in HEPES, pH 7.4, was incubated with DMS for 10 to 15 minutes. The stop solution and the labeled 12 base pair oligomer were added to this mixture. In a second sample, the DNA was added 5 minutes after the addition of the stop solution. The 0.1 M NaCl in HEPES, pH 7.4, was incubated with the DMS, the stop solution was added, and after 5 minutes the labeled 12 base pair oligomer was added. In a third sample, the stop solution was added 10 to 15 minutes after methylation of the DNA sample. The labeled 12 base pair oligomer in 0.1 M NaCl in HEPES, pH 7.4, was incubated with the DMS, and the stop solution was added after the

methylation occurred. **Figure 23** presents the autoradiogram obtained from this experiment.

The results showed similar cleavage patterns in all three samples. There was no difference in the stop solution reaction when added simultaneously with the DNA or 5 minutes before the DNA was added.

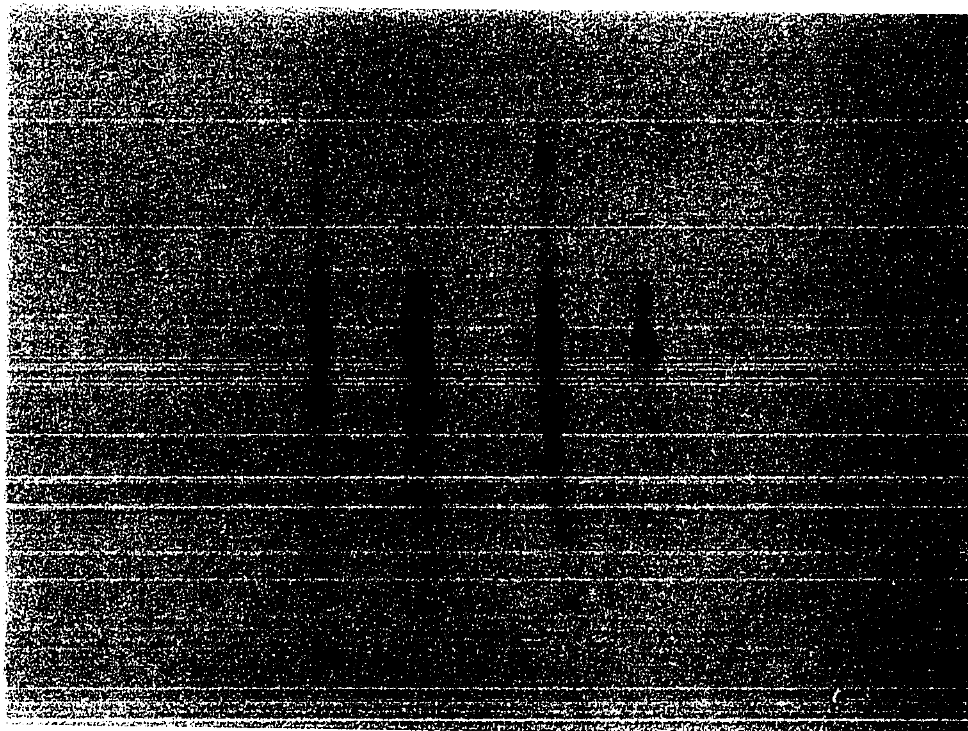


Figure 23: The effectiveness of the stop solution. The lanes are numbered from left to right. Lane 1, the DNA and the stop solution were added simultaneously (1.8×10^5 cpm); lane 2, the DNA was added 5 minutes after the stop solution (2.9×10^5 cpm); lane 3, the DNA was methylated and then the stop solution was added (2.9×10^5 cpm); lane 4, a labeled 12 base pair DNA (1.4×10^5 cpm)

Discussion

The Stability of Double Stranded DNA

The verification of the DNA state and the stability of the double stranded form is required for the methylation protection experiment. This is essential, since it is assumed that the grooves play a crucial role in the binding of protamine to DNA in a living cell (2), and grooves exist only in the double stranded form. The experiments and their results demonstrated that the 12 to 22 oligomers are stable in the double helix form (Figure 9). An increase in the absorbency of these oligonucleotides, and as a consequence, a calculated extinction coefficient ratio greater than one, indicated denaturation of the double helix DNA to single stranded chains, as was expected (16, 23). The results imply that these oligomers are stable as double stranded DNA and only forced denaturation melts them into single stranded DNA chains.

The 12 base pair oligomer presents at least one turn of the double helix, and was the shortest DNA chain (30% GC) to show stability in the double stranded form under these experiment conditions. The proposal that the central portion of the protamine is able to sufficiently fill the groove in one turn of the DNA (7) guided the choice of the 12 base pair DNA to study DNA-protamine binding.

On the other hand, the 10 base pair oligomer (30% GC content) seemed to show instability in the double stranded form. Half of the measurements resulted in a decrease in the absorbency measured after denaturation, yielding an extinction coefficient ratio which

fluctuates below and above one (Figure 9). Since the calculation is corrected for dilution, the reason for a ratio below one is not clear. The 10 base pair DNA was not used for the methylation protection experiment since its helix conformation is not stable under these conditions and thus, would not provide stable double stranded structures for the protamine P1 to bind.

The Dissociation of the DNA-Protamine Complexes

The dissociation of the DNA-protamine complexes occurred upon dilution in a 1-mL dissociation solution. The results showed that an equilibrium state was obtained for the complexes during the incubation time, since DNA-protamine complexes were captured on the filter paper at 0 time and the percentage of bound DNA at this time was the highest measured (Figures 10 and 15). A reduction in the amount of complexes, and therefore in the amount of bound DNA, was observed during a time period of 24 hours, indicating that dissociation in 0.1 M NaCl had occurred. The amount of non-specific binding observed was below 5% and the ability of these complexes to be dissociated revealed that the binding is reversible. The dissociation rate constant obtained ($k_2 = 1.1 \times 10^{-5}\ s^{-1}$) is within the same order of magnitude to the value reported previously (33), validating the model system used in the methylation protection experiment.

A goal in the development of the methylation and cleavage protocol was the use of the same solution to stop the methylation reaction and to dissociate the DNA-protamine complexes. Initial dissociation experiments were conducted with 3 M NaOAc solution, which has been used by Maxam and Gilbert in the stop solution for methylation (6). The

dissociation experiments using the 3 M NaOAc solution did not show any dissociation of DNA from the protamine even after one hour (Figures 11 and 12). A series of experiments to examine the pH effect on the dissociation in 3 M NaOAc and in 2 M NaCl (Figures 11 and 12), showed that the pH at 7.4 and 8.3 does not play a significant role in terms of dissociation. Therefore, it was concluded that the 3 M NaOAc solution could not be used as a dissociation solution in the methylation protection experiment. The use of $Mg(OAc)_2$ was considered as an alternative.

Record et al. (40) reported that Mg^{2+} competes for non-specific binding sites on the DNA, and since the binding of protamine to the DNA is not sequence-specific, this property of Mg^{2+} seemed to be useful for the dissociation of the DNA-protamine complex. In addition, Mg^{2+} was found by de Haseth et al. (41) to bind stronger than Na^{+} to the DNA. A solution with a stronger cation, such as Mg^{2+}, might be more effective for the dissociation process. Since the binding of the DNA to the protamine is driven (at least partially) by electrostatic forces (33) and the protamine is positively charged, a stronger cation solution may provide better displacement of the DNA. This may result in a more efficient dissociation solution.

The dissociation experiments were conducted with 3 M $Mg(OAc)_2$ solution and a constant percentage (34%) of bound DNA was observed with time (Figure 13). This solution failed to perform as a dissociation solution and no displacement of DNA was measured. It was discovered that the high viscosity of the 3 M $Mg(OAc)_2$ solution caused the filtration time to be relatively long, thus reducing the filtration method's effectiveness (33).

The surprisingly high residual binding after dissociation in 3 M NaOAc or 3 M $Mg(OAc)_2$ solutions (Figures 11, 12 and 13) led to a comparison of the non-specific binding to the resin and the filter paper with 0.1 M and 2 M NaCl solutions (Figures 11, 12 and 14). Both the NaOAc and the $Mg(OAc)_2$ solutions showed a significant amount of non-specific binding, indicating that the amount of DNA-protamine complexes was minimal. In any case, neither 3 M NaOAc nor 3 M $Mg(OAc)_2$ could be used as dissociation solutions, since they had very high non-specific bindings compared to the NaCl solutions (Table 3).

The 2 M NaCl solution was found to dissociate the DNA-protamine complexes in an efficient and rapid way (Figures 11 and 12) as has been proposed in the literature (8, 33). The results showed that at the initial time of dilution, about 10% of the DNA were still bound to the protamine and the same results were observed even after 60 minutes of dissociation (Figures 11 and 12), whereas the dissociation in 0.1 M NaCl under the same conditions increases with time from 56% at the initial time to 16% bound after 24 hours (Figure 10). Moreover, the dissociation using 50 μL of the DNA-protamine mixture was more efficient and extensive with 2 M NaCl than with the 0.1 M NaCl solution (Figure 15). The non-specific binding in the 2 M NaCl solution to the resin and to the filter were found to be very low, at a range of 2 to 5% (Figure 14), which is consistent with previous results (33).

The above results showed that the DNA-protamine complexes, which form in the presence of 0.1 M NaCl, can be efficiently dissociated with the 2 M NaCl solution. In addition, the low non-specific binding makes 2 M NaCl a good choice to use as the

dissociation solution for the methylation protection experiment. Since the stop solution in this procedure is the same as the dissociation solution, and since there is 1:1 dilution ratio between the stop solution and the sample, 4M NaCl solution was used to obtain the 2 M NaCl stop solution for the dissociation.

Another issue which needed to be examined is whether or not 2 M NaCl is an efficient stop solution for the termination of the methylation reaction on the DNA. However, the methylation and cleavage protocol and the gel electrophoresis separation had to be developed first, before the role of 2 M NaCl as a stop solution could be investigated.

Methylation and Cleavage

The results of the experiments using 12 and 22 base pair labeled DNA to test the quality of the sequencing gel and the films showed an identical migration distance for all the oligomers of the same size loaded on the gel in separate lanes. The separation of the bands and the migration distance of the various standards (Figure 17) verified the gel conditions to be appropriate for this experiment. Thus, the cleavage products can be separated and identified according to their migration distance.

Utilizing the original protocol (6), a 12 base pair DNA was processed, electrophoresed, and an autoradiogram was developed. A few bands appeared, which represented smaller fragments than 12 oligonucleotide chains, since they migrated further (Figure 16). These results verified that the methylation protection experiment to discover the exact location of the protamine in the DNA grooves, could be applied with slight

changes to the Maxam and Gilbert protocol (6).

The construction of the protocol started with the examination of the procedure using 5' end-labeled 12 base pair DNA with no protamine. According to the method proposed by Maxam and Gilbert (6), the first step in the process is methylation with the DMS reagent. Methylation with DMS occurs on N-7 of guanine in the major groove and on N-3 of adenine in the minor groove, and is known to be fivefold stronger on guanine than on adenine (12, 42). Since this free DNA is not protected by the protamine, all its sites are exposed and potentially can be methylated by the DMS. The next step was to stop the methylation reaction, and this was accomplished by adding the 2 M NaCl solution. All the subsequent steps were conducted according to the original protocol.

A diffusion of the radioactive material in the gel was observed (on the film) after a short time, but the bands became fuzzier and broader after 24 hours of exposure. To overcome this problem, the folders containing the gels and the films were wrapped and placed under a heavy weight at -70 °C to improve the contact between the gel and the film and to reduce the diffusion and the quenching, caused by the water molecules.

Although the bands on the autoradiograms appeared sharper than before, their intensity was too low. An increase in the [^{33}P]-labeled DNA amount used in each sample led to darker and sharper bands, since more labeled DNA was included in the individual bands. As a result, the cleavage pattern became visible. Clearly, when there is enough labeled DNA in the sample to begin with, there is more radioactive material in each band, enabling the detection of the short fragments obtained from the DNA cleavage.

Even though bands appeared on the film with good intensity, large amounts of the

radioactive material were still found at the origin and did not migrate into the gel. This could have been a result of aggregation or incomplete denaturation of the DNA. The aggregation causes the formation of DNA clusters, which are much too bulky and charged to migrate. Incomplete denaturation prevents the separation of the double stranded DNA and thus, the cleaved fragments still remain bound to other strand(s) by hydrogen bonds, again inhibiting migration. This problem was avoided by placing the samples in a water bath at 90 °C for 1 minute before loading on the gel. The heat induces thermal denaturation and thus melts the double stranded DNA or the clusters to single strands (16), which are maintained in a single stranded form by the formamide in the loading buffer.

At this stage, each and every step of the protocol was tested and the results (Figure 16) revealed that the modified procedure was appropriately adapted to the DNA-protamine system.

Localization of Protamine Binding on the DNA

The main purpose of this project was to determine the binding site of the protamine on the DNA by a methylation protection experiment (5, 6). The methylation protection experiment (5) is based on the idea that the protamine binds to the DNA, covering the binding site as it binds. The methylation occurs on the exposed site, and the cleavage occurs only at the methylated bases (5, 6). A comparison between the bound DNA and the free DNA, in terms of cleavage sites and fragment size, should provide a clear picture as to whether any of the sites are blocked by the protamine and if so, in

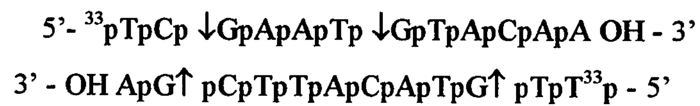
which of the grooves the protamine is bound.

The sequence of the 12 base pair oligomer is known and both the strands are labeled at the 5' end. A scheme of the sequence and the cleavage sites is presented in **Figure 24**. The cleavage pattern was expected to produce intense radioactive bands at 2, 6, and 10 nucleotides in size, since these are the fragments provided by guanine sites in the sequence (positions 3, 7, and 11, respectively). Lighter intensity bands at 3, 4, 6, 8, 10, and 11 nucleotides in size were expected, since these locations present the adenine sites in the sequence, 4, 5, 7, 9, 11, and 12, respectively. The fragments at 6 and 10 nucleotides in size are contributed by both strands of the 12 base pair oligomer, thus these bands should appear with more intensity than others. The bound DNA was expected to produce less bands than the free DNA, because it was believed that some of the methylation sites would be covered by the protamine.

The results from the methylation protection experiment presented four bands for the bound DNA, corresponding to 12, 10, 8 and 4 oligonucleotides in size as determined from the migration of standards (Table 5). Moreover, the same pattern was presented by the free DNA as well (Figures 19 and 20).

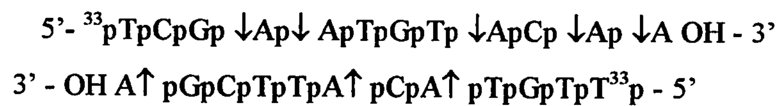
The experiment testing the stop solution's effectiveness (Figure 23) offered one possible explanation for the similar cleavage patterns between the bound and the free DNA. The results clearly showed that the methylation reaction does not terminate immediately with the addition of the stop solution, but rather at a much slower rate. Therefore, it is possible, that the DNA continued to be methylated by the DMS after the

The guanine cleavage sites:



The cleavage pattern is: 2, 6, and 10 nucleotides in size from the 5' end of a strand.

The adenine cleavage sites:



The cleavage pattern is: 3, 4, 6, 8, 10, and 11 nucleotides in size.

Figure 24: The cleavage sites at guanine and adenine and the labeled fragments obtained from the cleavage.

complexes were dissociated. When this happens, the cleavage pattern of the bound DNA would be similar to that of the free DNA.

The stop solution seemed to be an important factor in the determination of the results. However, an equivalent methylation and cleavage pattern (in a presence of an effective stop solution) could be obtained as a result of several other possibilities. It may be that the protamine does not bind directly in one of the grooves but forms a bridge over the grooves by contacting only the phosphodiester backbone. Thus no preferential protection of either groove would be provided. In agreement with these results, Mirzabekov (12) reported that protamines are unlikely to be bound in the grooves, and thus the methylation of the bound and the free DNA have the tendency to be very similar. Bianch et al. (43) reported that human protamines (P1 and P2) do not seem to bind in any of the grooves and they interact mainly with the phosphodiester backbone. Even though binding to the grooves, as suggested by Pogany (2) and Balhorn (7), provides an explanation for the incredible condensation, the results of these methylation protection experiments support the binding to the phosphodiester backbone, which can affect the hydration state of the complex and thereby lead to condensation.

There is a possibility, as proposed by Suau and Subirana (3) and Balhorn (7), that the DNA-protamine complex is not as closely bound as may have been expected, and that intervals can be formed between the protamine and the double helix DNA. The dimethyl sulfate, which is a small molecule, could find its way under the protamine within the opening to the DNA bases to methylate them. The cleavage pattern of the free and the bound DNA would appear exactly the same in this case, since the protamine would not

really protect or cover the DNA sites and would not prevent methylation upon binding. The protamine may still lie in the grooves (3, 4, 7, 34, 35) but the binding may flex, allowing the DMS reagent to methylate the bases under the protamine.

Some of the models (3, 4, 7, 34, 35) proposed multiple binding sites for protamines on one DNA helix. The protamines bind one next to another, and this organization provides a protection of their binding region, thus DNA is less accessible to reagents or enzymes. The complexes in this study are based on isolated DNA-protamine complexes, in which one protamine is assumed to be bound to one DNA segment, containing one helical turn. There is a possibility of solvent access to the grooves even when the protamine is located within a specific groove, through the binding site ends. The lack of cooperativity with other protamines may lead to the exposure of methylation sites, since there are no other protamines bound that would hinder this access.

Another explanation for the similar cleavage pattern between the free and the bound DNA can be related to the lifetime of the DNA-protamine complex. The half-life of the DNA-protamine complex under these conditions (0.1 M NaCl, 50 mM HEPES) is approximately 117 minutes, and 90% of the these complexes should remain intact for 17.7 minutes (33). However, it is possible that during the methylation period, which is recommended to occur between 10 to 15 minutes, the complexes partially dissociate and methylation occurs on the dissociated DNA, which would be fully exposed and unprotected. The results from such a process would yield similar patterns of cleavage.

Another possibility is also apparent. If the protamine binds equally to the minor and the major grooves, the DNA methylation would be similar to the methylation obtained

if the binding occurs outside the grooves. On average, both the grooves would be exposed for methylation and the cleavage pattern would appear similar to that of the free DNA.

Many proposed models (3, 4, 7, 34, 35, 43) for the DNA-protamine complex binding mode suggested that the protamine is also used for connecting neighboring DNA helices. Suau and Subirana (3) presented a binding model, in which one protamine is bound to the minor groove of a few DNA helices, thus connecting them into a condensed structure (Figure 4b). Warrant and Kim (34) proposed a model in which the protamine is bound to the major groove of one helix and connects two neighboring DNA helices by binding to their minor groove (Figure 6). According to Balhorn's model (7), the protamine lies in the minor groove and connects the neighboring helices through the major groove (Figure 7). Bianchi et al. (43) presented evidence for the binding of one protamine to a few DNA molecules by the formation of protamine bridges between them. The various models attempt to simulate the living system and provide a possible mechanism for the condensation of the DNA and the connections between DNA helices. The DNA-protamine system examined here is limited to a single protamine complexed with a 12 base pair oligomer. There should not be any connection between adjacent complexes and any structures, which involve more than one DNA molecule. An isolated system as used here in the experiments, may cause a totally different binding mode, which is specific to this single complex system.

Various possible explanations had been given for the observed similarity between the cleavage patterns of the bound and the free DNA. These explanations ranged from the ineffectiveness of the stop solution, through the binding of the protamine in locations other

than the grooves, and the uniqueness of the isolated complex system. The remaining issue is to try and understand the obtained cleavage patterns from the methylation protection experiments.

It is important to ensure that the methylation of the free DNA was behaving as expected, that is preferential methylation of guanine (6). The sequence of the 12 base pair oligomer is known, and accordingly the methylation and the cleavage pattern was expected to yield bands corresponding to 2, 3, 4, 6, 8, 10 and 11 oligomers in size. Only oligomers of 4, 8, 10 and 12 in size were registered on the autoradiogram, a cleavage pattern which appears to be more consistent with methylation of A rather than G.

The determination of the cleaved products' size is based on the comparison of their migration distances with those of the standards. However, in order to compare accurately, one has to consider the difference in the mass-to-charge ratio between the standards and the cleavage products. The cleaved products have an additional phosphate group at the 5' end as a result of the cleavage reaction (Figure 3), causing a charge increase, which may influence the migration distance. The uncleaved [^{33}P] 12 base pair DNA, however, has an identical mass-to-charge ratio as the standards. They both contain an OH group at the 3' end, and yield comparable migration distances. **Figure 25** presents the correlation between the oligonucleotide's size and its mass-to-charge ratio.

The initial comparison of the migration distances between the standards and the cleavage products was based on the assumption, that the mass-to-charge ratio is constant with the increase of the nucleotide size. From analysis of the data presented in Figure 25, it is obvious that for the standards this assumption is valid from about 5 nucleotides in size

and larger, however, for the cleaved products this assumption is valid only from about 10 nucleotides in size. Since this experiment deals with the cleavage of 12 base pair oligomers, the determination of the fragments' size cannot be based only on comparison to the standards.

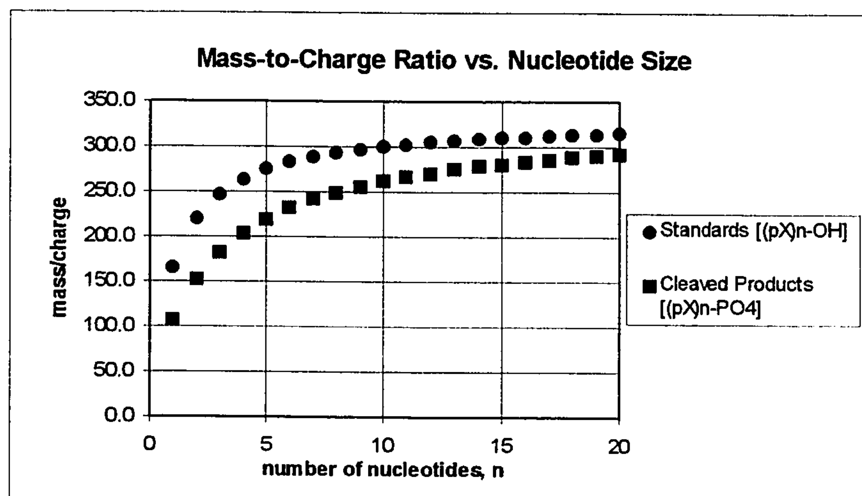


Figure 25: The correlation between the oligonucleotide's size and its mass-to-charge ratio for standards and cleaved products.

Conclusions

The research work and the results obtained led to several conclusions. A complete protocol for the methylation protection experiment of the DNA-protamine complexes has been developed. During the process it was found that oligonucleotides of 12 to 22 base pairs in size that are at least 30% GC rich are stable in the double helix form of DNA, whereas the 10 base pair oligomer (30% GC content) is unstable as double stranded DNA under the experimental conditions used (0.1 M NaOH in 50 mM HEPES, room temperature).

A stop solution containing 2 M NaCl, 1 mM β-mercaptoethanol and 100 μg/mL DNA is not efficient for the termination of the methylation reaction. However, 2 M NaCl solution was found to be the best dissociation solution for the DNA-protamine complexes, among the solutions examined. $Mg(OAc)_2$ and NaOAC produce very high non-specific binding of DNA to the resin and should not be used to initiate the dissociation of the complexes.

Poor stop solution performance resulted in similar patterns of methylation and cleavage of the free DNA and the bound DNA. Additional situations which could lead to similar methylation and cleavage products of free and bound DNA are:

1) The protamine does not bind in the grooves, but lies outside of them;

2) Binding occurs by neutralizing the phosphates and laying on top of the groove but does not disrupt solvent access to the bases;

3) The lifetime of the DNA-protamine complex is too short and the dissociation occurs during the methylation period.

The binding of the isolated DNA-protamine complex may be different from the binding in the living system. Since there is no connection between neighboring DNA helices and only one protamine is bound to the 12 base pair DNA, greater accessibility of the DNA to the methylation reagent is possible and thus methylation and cleavage at all sites.

Future Work

An important step in the methylation and cleavage protocol has to be examined before the investigation of any other applications. The search for an effective stop solution, which would provide immediate termination of the methylation reaction produced by the DMS reagent, is essential. Once finding the appropriate stop solution, the methylation reaction of the DNA-protamine complexes reported here should be repeated. In order to obtain a complete and satisfying picture of the DNA-protamine system, further investigation using different footprinting methods, such as methylation protection with larger methylating agents, methylation interference, ethylation and hydroxyl radical, should be conducted. Moreover, the use of several DNA sequences and different forms of DNA can provide information regarding the binding to the various grooves. Perhaps the role of different structural features may become apparent with such an approach. Another aspect of the work which would contribute to the understanding of the DNA-protamine system may be investigated by utilizing the same experimental design with various protamines, such as P1 protamine from other mammals or protamine P2.

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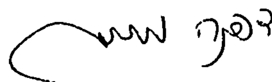

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